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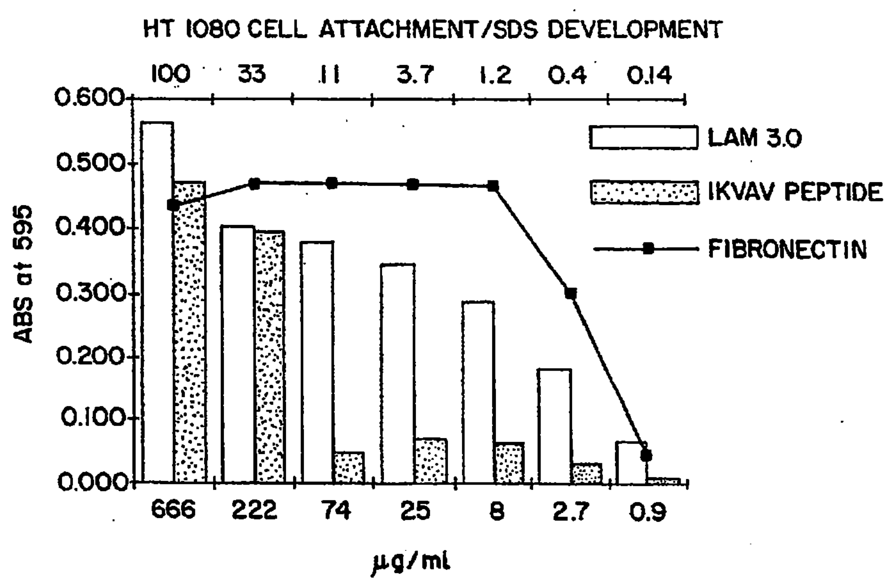

(57) Abstract

Thermoplastics interdispersed with a variety of functional thermostable proteins and methods of making such thermoplastics are provided. The disclosure demonstrates that certain proteins can maintain functional integrity through exposure to plastic thermomolding. The proteins are exposed to the heating and molding/extrusion/casting process and are hence present on the formed plastic surface and at a depth below the plastic surface. The proteins contained in the disclosed compositions retain functional properties or binding specificities through the heating and molding/extrusion/casting processes. Preferred thermostable protein used in the disclosed compositions include silk-like protein polymers, particularly ProNectin®F. The disclosed methods and compositions find use in many applications where plastics containing functional thermostable proteins are desired, in particular, cell cultureware.

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PROTEIN-ENRICHED THERMOPLASTICS

CROSS-REFERENCE TO RELATED APPLICATIONS

This Application is a continuation-in-part of Application Serial No. 609,716 filed November 6, 1990, which is a continuation-in-part of Application Serial No. 269,429 filed November 9, 1988, which is a continuation-in-part of Application Serial No. 114,618 filed October 29, 1987, now U.S. Patent no. 5,243,038, which is a continuation-in-part of Application Serial No. 927,258, filed November 4, 1986, and claims priority to PCT 89/05016, filed November 7, 1989.

INTRODUCTION

Technical Field

The field of this invention is thermoplastics incorporating thermostable polypeptides.

Background

The immobilization of functional polypeptide provides an enormously broad range of applications from medical diagnostics, medical implants, chemical separations, chemical sensors, cultureware, etc. Because of their relatively low reactivity and expense, plastics are the most common solid substrates for protein immobilization. Heat is often used in the fabrication of useful articles from thermoplastic resins and elastomers as well as thermosetting resins and elastomers. Typically the heating and extrusion/molding process requires temperatures in the 100 - 400°C range and often much higher.

Most polypeptides are irreversibly denatured and lose their functional properties at temperatures above about 50 - 60° C. The exceptions are polypeptides of a few thermophilic bacteria surviving the environs of hot springs and undersea thermal vents

which have recently been shown to have heat stabilities up to 100° C. To accommodate the thermal lability of polypeptide function, immobilization with plastic is accomplished by attaching a selected polypeptide to a pre-formed plastic surface either covalently, usually by chemical activation of the substrate surface, or non-covalently, usually called adsorption.

The vast majority of plastics have hydrophobic surfaces. For many applications such as cell culture and immunodiagnostics, it is critical to have a hydrophilic surface that aqueous fluids will wet. Current treatments commercially employed include plasma treatment to cause the formation of ionizable chemical groups on the surface, oxidation under conditions of irradiation, or by deposition of surfactants on the surface.

Accordingly, there are a number of deficiencies with current polypeptide immobilization methods and compositions. Solvent, vapor or powder deposition are labor, time and material intensive. Surface coatings are subject to mechanical wear and erosion, chemical modification or degradation, and removal by the action of solvents. Some articles, such as bottles, are difficult to surface coat. And, especially in the case of cultureware, post-manufacture sterilization steps are often required.

Relevant Literature

Wood and Gadow (1983) J Clin Chem Clin Biochem 21, 789-797 review immobilization of proteins on solids; Ponnuswamy et al (1982) Int J Biol Macromol 4, 186-190; Dale et al and Wampler et al (1992) ACS Symp Ser 498 (Biocatal Extreme Temp), 136-152 and 153-173; Finkelstein and Reva (1991) Nature 351, 497-499; Goodenough and Jenkins (1991) Biochem Soc Trans 19, 655-662; Wozniak et al (1990) Crystallogr Model Methods Mol Des, Ed. Bugg and Ealick, NY NY; Mathews et al (1987) Proc. Natl. Acad. Sci. 84, 6663-6667 discuss protein compositional parameters relating to thermal stability; Santoro et al (1992) Biochemistry 31, 5278-5283; Lucy and Lee (1987) Biochemistry 26, 7813-7819; MacLeod et al (1984) Res Discl 244, 380 describe agents which affect the thermal stability of proteins.

SUMMARY OF THE INVENTION

The present invention relates to the finding that certain polypeptides can maintain functional integrity through exposure to plastic thermomolding. Accordingly, thermoplastics containing a variety of functional thermostable polypeptides and methods of making such polypeptide containing thermoplastics are provided. The polypeptides are exposed to the heating and molding/extrusion/casting process and are hence present on the formed plastic surface and at a depth below the plastic surface. The polypeptides contained in the disclosed compositions retain functional properties

through the heating and molding/extrusion/casting processes. Preferred thermostable polypeptide used in the disclosed compositions include silk-like protein polymers, particularly ProNectin®F. The disclosed methods and compositions find use in many applications where plastics containing functional polypeptides are desired, in particular, cell cultureware.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1. is a graph showing HT 1080 Cell Attachment/SDS Development.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The invention provides methods and compositions relating to thermoplastics containing functional thermostable polypeptides. These compositions present numerous advantages over conventionally coated plastics: increased durability of the surface activation to mechanical wear and erosion or the action of solvents, increased resistance to chemical modification or degradation, lower costs of production, broader range of articles for manufacture, *etc.*

The thermoplastics of the invention are broadly defined to encompass a wide variety of chemical compositions. By "plastic" is meant a polymeric material, preferably organic, of large molecular weight, usually between 10^3 and 10^6 MW, which can be shaped by flow. In addition to the base resin, formulations of thermoplastic, including thermosetting polymers, for use in thermomolding applications may include a variety of additives such as stabilizers, accelerators, retardants, antimicrobials, lubricants, fillers, plasticizers, pigments, *etc.* However, in certain embodiments, some additives interfere with the presentation or function of polypeptides at the surface. For example, as shown below, zinc stearate can exhibit a blooming effect whereby surface protein is masked by migrating small molecular weight compounds. Similarly, the compatibility of any selected additive with the methods and compositions disclosed herein is readily determined.

Preferred plastics are amenable to injection molding (they are melt processable at less than about 300° C and have Tg's of less than about 200° C), and are least reactive toward the selected thermostable protein, in particular, under injection molding conditions. Plastics with a potential of engaging in amidation or transamination reactions, such as polyamides or polyesters are less desirable. Most preferred plastics include polystyrene, polypropylene, polyethylene and polyvinyl chloride. Other useful plastics include polyvinylidene fluoride, polyvinylidene chloride, acrylonitrile butadiene styrene (ABS), styrene acrylonitrile (SAN), and polyacrylonitrile (PAN). Plastics which find use but also entail chemical reactivity toward some protein groups include

both aromatic and aliphatic polyamides and polyimides, polyacrylates, polymethacrylate esters, polydioxanone, polyanhydrides, and polyesters such as polycarbonate, polybutylene terphthalate, polyethylene terphthalate, polyglycolic acid and polylactic acid and PLGA copolymers, polyhydroxybutyrate (PHB), polyurethanes, and homopolymers and copolymers of polyvinyl alcohol esters such as polyvinyl acetate and ethylene vinyl acetate. Plastics may also include rubbers such as polysiloxanes, polybutadienes, and neoprenes. However, any plastic capable of thermal extrusion/casting/molding may find use herein.

As numerous applications of the disclosed compositions involve contact with viable biological cells or tissue, biocompatible plastics are especially preferred. Biocompatible plastics are typically non-toxic, biochemically inert, and nonproductive of undesired immune responses when used *in vivo*. Exemplary biocompatible plastics include polycaprolactone, polycarbonate, polydimethylsiloxane (silicone rubber), polydioxanone, polyether urethane, polyethylene and polyethylene terphthalate, polyglycolic acid and polylactic acid and PLGA copolymers, polyhydroxyethyl methacrylate (HEMA), polymethylmethacrylate (acrylic), and polyvinyl chloride (PVC). Also useful are biodegradable plastics, preferably plastics that degrade under physiological conditions, including polycaprolactone, polydioxanone, polyglycolic acid and polylactic acid and PLGA copolymers, and polyanhydrides. Such plastics are especially useful in diagnostics, therapeutics, and environmental monitoring where time-release of the contained proteins or where subsequent removal of the plastic is inconvenient.

The plastics which are mixed with a thermostable polypeptide according to the present invention may be obtained in any convenient form and are generally commercially available or readily obtained by those of ordinary skill in the art. Generally, a polymerized form is preferred; though, where the polymerization conditions are compatible with the preservation of polypeptide functional integrity, monomers may be used. The compatibility of the polypeptide with the polymerization depends in large part on the reactivity of the particular amino acid composition of the selected protein in the polymerization reaction. For example, vinyl polymerization finds some use while condensation polymerization of polyesters and polyamides is less useful.

The thermostable polypeptides are interdispersed or present on the surface of the plastics as well as being contained within the plastics of the invention. By interdispersed is meant that at least some of the protein is found beneath the surface of the plastic. Prior art plastics have been surface coated with polypeptide from solution or dispersion in liquids or in powder form. By incorporating the thermostable protein

before or during the thermomolding process rather than as a post-molding coating step, the present invention provides subsurface protein as opposed to solely a surface coating layer on the plastic. Accordingly, the disclosed materials do not have a clearly defined polypeptide-plastic interface. In a preferred embodiment, the thermostable polypeptide is present throughout most of the volume of the thermomolded plastic object. The thermostable polypeptide is present in the range of about 1-10,000 ppm; generally, at less than 2,000 ppm, preferably less than 500 ppm, more preferably less than 100 ppm. Polypeptide is usually detectable at concentrations greater than 1 ppm, preferably greater than about 10 ppm, at a depth of .01 μm, preferably .1 μm, and more preferably at least about 1 μm below the surface of the plastic.

By functional thermostable polypeptide is meant a protein, polypeptide, or peptide that at least partially retains the native protein, polypeptide or peptide's structure (primary, secondary or tertiary) and retains one or more specific functions of the native polypeptide after exposure to the thermal molding/extrusion/casting conditions described herein. Exemplary retained specific functions include catalytic or enzymatic activity, binding specificity, covalent, ionic, or non-covalent interactions with the environment for example, chemical conjunction with various reagents, and to a less preferred extent, defined wettability, ionic conductance, etc. By binding specificity is meant a molecular spatial orientation specifically recognizable by, for example, a protein receptor. Examples include cellular ligands (an epitope to which cell surface receptors bind), immunological epitopes (an epitope to which an antibody binds), sugar moieties (an epitope to which a lectin may bind), peptide-specific epitopes (an epitope to which a peptide - usually a synthetic peptide of 3-60 residues screened for component-specific binding from peptide libraries or denovo design - bind), *etc.*

Functional thermostable polypeptides are readily identified by functional assays of processed plastic containing the polypeptide, commonly cell culture or protein or ligand binding assays, including those used to assess the function of proteins immobilized on surfaces using previous methods in the art, although many other assays may be employed, depending upon the function, which will be readily recognized by those skilled in the art. For example, catalytic activity, binding specificity, physical chemical properties, *etc.* are all readily tested by conventional immunoassays, spectroscopy, microscopy, *etc.* Candidate polypeptides for the above functional assays are selected by the potential market of their intended application and predicted functional thermostability. Indications of functional thermostability include resistance to decomposition or irreversible denaturation so as to lose their desired function under the plastic processing conditions; inclusion of relatively few amino acids susceptible to high temperature chemical modification or cleavage such as lysine and aspartic acid;

inclusion of relatively high proportions of amino acids known to be associated with thermally stable proteins such as arginine, alanine, threonine, asparagine, isoleucine, or glutamic acid; structures with a high degree of intrachain bonding such as hydrogen bonds or covalent cross-links; hydrogen bonded antiparallel beta sheets with a high accessible surface area; and activity or functionality contained in a single chemically contiguous protein or peptide chain.

The polypeptides are typically of large molecular weight, usually more than about 6 kD, preferably more than 25 kD, more preferably more than 50 kD. However, polypeptides of at least 3, preferably at least about 6 more preferably at least about 12, most preferably at least about 24 amino acids in length may also be employed. Preferred thermostable polypeptides include structural proteins such as elastin-, collagen-, keratin-, and silk-type proteins, preferably, proteins derived from thermophilic bacteria such as Sulfolobus solfataricus and Thermus aquaticus (enzymes such as proteases, DNA polymerases, lipases, and metabolic enzymes are especially useful and more preferably, synthetic protein polymers, particularly proteins designed with silk-like protein, SLP blocks (SLPF or FCB-SLPIII (fibronectin), SLPL (laminin), SLPC (cystine), SLP3, SLP4, and SELPs (elastin) as described in US Patent Application Nos. 609,716 and 114,618, and peptides designed with SLP blocks (peptide 92.7: KKMGAGAGSGAGAGSGAAVTGRGDSPASAAGYGAGAGSG-AGAGS), where ProNectin®F (PnF, SLPF or FCB-SLPIII) is most preferred. The polypeptides may be natural, chemically synthesized, or recombinant proteins, including modified forms such as mutants and fusion products, and also including modifications against thermally induced degradation or denaturation, for example, pegylation.

The genes of the subbject invention comprise multimers of DNA sequences encoding the same amino acid sequence unit, where two or more multimers encoding different amino acid units may be joined together to form a block copolymer. The individual units will have from 3-30 amino acids (9-90 nt), more usually 3 or 4 to 25 amino acids (9-75 nt), particularly 3 or 4 to 15 amino acids (9-45 nt), or particularly 3 or 4 to 9 amino acids (9-27 nt), usually having the same amino acid appearing at least twice in the same unit, generally separated by at least one animo acid. The units of the multimer coding for the same amino acid sequence may involve two or more nucleotide sequences, relying on the codon degenracy to achieve the same amino acid sequence.

Peptide polymers having intervening sequences may provide for chemically active amino acids for chemical crosslink sites, which may serve to covalently attach functional peptides, synthetic or natural polymers or ptoteins, non-amino acid molecules, and the like. The intervening sequence may be a naturally occurring

sequence or a modified naturally occurring sequence. Naturally occurring sequences may be derived from a wide variety of sources with a variety of functions. Such sequences may be a cellular growth inhibitor sequence, *e.g.* from Tenascin; cell growth promoting attachment factors, *e.g.* from fibronectin, RGD-, -REDV-, laminin B1 -YIGSR-; bacterial adhesive -SLF-, -ALF-; growth hormones and insulin; inclusion sequences (GAGC and GCCV, which provide systems for attachment and crosslinking; VSPD, VCPD and DPGK, which provide an underlining structure).

By thermomolding is meant that the plastic is exposed to heat in the fabrication process. Generally, heat is used to melt the plastic for molding, and, in the present invention, for distributing polypeptide beneath the surface of the plastic. Thermomolding refers to any method of heating and forming the plastic and includes extrusion, injection molding, thermoforming, thermosetting, compression molding, etc. Extrusion includes die, cast film, sheet, profile and wire processes. Injection molding is preferred for most articles, especially cultureware, and includes structural foam, blow molding (useful for producing roller bottles), and rotational molding. Less preferred embodiments include reaction injection molding because of potential cross-reactivity with the polypeptide.

Thermomolding is generally performed according to conventional methods. This molding step is usually performed at temperatures in excess of 60° C, preferably in excess of 100° C and more preferably in excess of 140° C; though temperatures in excess of 200° C and 300° C also find use herein. The manufacturing temperature is determined by the character of the plastic resin as well as the thermostability of the polypeptide. Thermal stability boundaries are readily determined using the methodologies described below. A variety of methods may be employed to enhance the thermal stability of the polypeptides under the thermomolding conditions, such as the addition of organic acids, divalent cations, zwitterions, or saccharides, and decreases in the moisture content of the mixture of polypeptide and plastic prior to thermomolding. As exemplified below, the IKVAV presenting domain of SLPL3.0 which was inserted into SLP3 contains a lysine, which is less reactive when protonated. Residual formic acid was left in the coated polystyrene powder coated with SLPL3.0 and enhanced performance was observed after thermomolding compared to the untreated control. Similarly, Ca^{2+} and glycine were shown to confer additional resistance to thermal degradation in the case of polystyrene powder coated with PnF.

The polypeptide may be added at a variety of stages of the manufacturing process so long as heat is applied during or after the addition of the protein. Thus for example, polypeptide (in solution or dry) may be mixed with commercial resin pellets before heating and extrusion, with a melt before, in, or after the final metering of the

extruder, *etc.* Dispersing agents known to those skilled in the art may be used to enhance mixing of polypeptides into the plastics under thermomolding conditions. The polypeptide may be added before extrusion and the extruded ribbon reheated while being compressed in to the final article. Alternatively, the polypeptide can be applied to the surface of the extruded ribbon and then compression molded to form the final article. For instance, a film can be coated and then heated in a mold to form a microtiter plate.

The thermomolded protein-enriched plastics may take a wide variety of forms depending on the intended application. Preferred forms include sheets, membranes, beads, fibers, hollow fibers, tubes and formed vessels. Preferred vessels include tissue culture matrices such as petri dishes, culture flasks, roller bottles and microtiter-type plates. The plastics may be solid, porous, or semiporous and may be made bio- or environmentally degradable by techniques described herein or otherwise known to those skilled in the relevant art.

The polypeptide-enriched thermoplastics of the present invention find a wide variety of uses, especially in the chemical, biotechnology, and health care industries. The materials find use, for example, in separation techniques such as chromatographic or filtration matrices; in therapeutic techniques such as controlled drug delivery (*e.g.* transdermal skin patches and osmotic pumps), sutures, catheters, etc.; in diagnostic techniques; and in tissue culture matrices.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Construction of SLP-F9

Two oligonucleotide strands were synthesized and purified as described in the Methods section of the US Application No. 07/609,716.

```
             (PstI)       SnaBI                                    (PstI)
i)    5'-      CGCTACGTAGTTCTGCCACGTCCGGTATGTTTCGAAAAAGCTGCA   -3'
ii)   3'-          ACGTGCGATGCATCAAGACGGTGCAGGCCATACAAAGCTTTTTCG-5'
```

These oligonucleotide strands were annealed and ligated with plasmid pSY1304 which had been digested with PstI REN.

The product of this ligation reaction was transformed into *E. coli* strain HB101. Plasmid DNA from transformants was purified and digested with BanI; clones containing inserts of the correct size were digested with BsaAI REN to determine the restriction pattern. Plasmid DNA from correct clones was sequenced. Plasmid pPT0272 (shown in Table 1) contained the desired SLP-F9 monomer sequence.

Table 1.

```
GGT GCC GGC AGC GGT GCA GGA GCC GGT TCT GGA GCT GGC GCG GGC TCT GGC GCG GGC
G   A   G   S   G   A   G   A   G   S   G   A   G   A   G   S   G   A   G

GCA GGA TCC GGC GCA GGC GCT GGT TCT GGC GCA GGG GCA GGC TCT GGC GCA GGA GCG
A   G   S   G   A   G   A   G   S   G   A   G   A   G   S   G   A   G   A

GGG TCT GGA GCT GCA CGC TAC GTA GTT CTG CCA CGT CCG GTA TGT TTC GAA AAA GCT
G   S   G   A   A   R   Y   V   V   L   P   R   P   V   C   F   E   K   A

GCA GGC TAT GGA GCT GGC GCT GGC TCA GGT GCT GGA GCA GGA AGC GGA GCG
A   G   Y   G   A   G   A   G   S   G   A   G   A   G   S   G   A
```

Plasmid DNA from pPT0272 was digested with BanI REN and the digestion fragments were separated by agarose gel electrophoresis. The SLP-F9 gene fragment, 222 bp, was excised and purified by NACS column (see Example 1 of US Appl No. 07/609,716). The purified fragment was ligated with plasmid pSY1262 which had been

digested with REN BanI. The product of this ligation reaction was transformed into E. coli strain HB101. Transformants were selected for resistance to kanamycin. Plasmid DNA from individual transformants was purified and analyzed for increased size due to SLP-F9 multiple DNA insertion. Several clones were obtained ranging in size from 1 kbp to 4 kbp. One clone pPT0275, with an insert of approximately 2.7 kbp was chosen for expression and protein analysis.

SLP-F9 Expression

An overnight culture which had been grown at 30° C was used to inoculate 50 ml of media contained in a 250 ml flask. Kanamycin was added at a final concentration of 50 µg per ml and the culture was incubated with agitation (200 rpm) at 30° C. When the culture reached an OD$_{600}$ of 0.8, 40 ml were transferred to a new flash prewarmed at 42° C and incubated at the same temperature for approximately 2 hours. The cultures (30° and 42°) were chilled on ice and OD$_{600}$ was taken. Cells were collected by centrifugation divided in 1.0 OD$_{600}$ aliquot and used to perform dot blot and western analysis using SLP antibodies. For purification and amino acids analysis larger cultures were used.

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pPT0275          SLP-F9          945 AA          75,561 MW
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MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGS (GAGAGS)$_6$

[GAA RYVVLPRPVCFEKAAGY (GAGAGS)$_9$]$_{11}$

GAARYVVLPRPVCFEKAAGY (GAGAGS)$_2$ GAGAMDPGRYQLSAGRYHYQLVWCQK

Construction of SLP-L3.0

An additional two oligonucleotide strands were synthesized as described in the Methods section of US Appl No. 07/609,716.

```
         (PstI)      ClaI                           (PstI)
iii) 5'-      CCGGGTGCATCGATCAAAGTAGCTGTTAGCGCCGGACCGTCTGCA-3'
iv)  3'-ACGTGGCCCACGTAGCTAGTTTCATCGACAATCGCGGCCTGGCAG      -5'
```

These oligonucleotide strands were annealed and ligated with plasmid pSY1304 which had been digested with PstI REN.

The product of this ligation reaction was transformed into E. coli strain HB101. Plasmid DNA from transformants was purified and digested with BanI; clones

containing inserts of the correct size were digested with StuI and ClaI RENs to determine the restriction pattern. Plasmid DNA from correct clones was sequenced. Plasmid pPT0271 (shown in Table 2) contained the desired SLP-L3.0 monomer sequence.

Table 2.

GGT	GCC	GGC	AGC	GGT	GCA	GGA	GCC	GGT	TCT	GGA	GCT	GGC	GCG	GGC	TCT	GGC	GCG	GGC
G	A	G	S	G	A	G	A	G	S	G	A	G	A	G	S	G	A	G
GCA	GGA	TCC	GGC	GCA	GGC	GCT	GGT	TCT	GGC	GCA	GGG	GCA	GGC	TCT	GGC	GCA	GGA	GCG
A	G	S	G	A	G	A	G	S	G	A	G	A	G	S	G	A	G	A
GGG	TCT	GGA	GCT	GCA	CCG	GGT	GCA	TCG	ATC	AAA	GTA	GCT	GTT	AGC	GCC	GGA	CCG	TCT
G	S	G	A	A	P	G	A	S	I	K	V	A	V	S	A	G	P	S
GCA	GGC	TAT	GGA	GCT	GGC	GCT	GGC	TCA	GGT	GCT	GGA	GCA	GGA	AGC	GGA	GCG	GGT	GCC
A	G	Y	G	A	G	A	G	S	G	A	G	A	G	S	G	A	G	A

Plasmid DNA from pPT0272 was digested with BanI REN and the digestion fragments were separated by agarose gel electrophoresis. The SLP-L3.0 gene fragment, 222 bp, was excised and purified by NACS column (see Example 1 of US Appl No. 07/609,716). The purified fragment was ligated with plasmid pSY1262 which had been digested with REN BanI. The product of ligation reaction was transformed into E. coli strain HB101. Transformants were selected for resistance to kanamycin. Plasmid DNA from individual transformants was purified and analyzed for increased size due to SLP-L3.0 multiple DNA insertion. Several clones were obtained ranging insize from 1 kbp to 4 kbp. One clone pPT0278, with an insert of approximately 2.9 kbp was chosen for expression and protein analysis.

SLP-L3.0 Expression

An overnight culture which had been grown at 30° C was used in inoculate 50 ml of media contained in a 250 ml flask. Kanamycin was added at a final concentration of 50 μg per ml and the culture was incubated with agitation (200 rpm) at 30° C. When the culture reached an OD_{600} of 0.8, 40 ml were transferred to a new flask prewarmed at 42° C and incubated at the same temperature for approximately 2 hours. The cultures (30° and 42°) were chilled on ice and OD600 was taken. Cells

were collected by centrifugation divided in 1.0 OD_{600} aliquot and used to perform dot blot and western analysis using SLP antibodies. For purification and amino acids analysis larger cultures were used.

pPT0278 SLP-L3.0 1019 AA 75,639 MW

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGS $(GAGAGS)_6$
$[GAAPGASIKVAVSAGPSAGY\ (GAGAGS)_9]_{12}$
GAAPGASIKVAVSAGPSAGY $(GAGAGS)_2$ GAGAMDPGRYQLSAGRYHYQLVWCOK

<u>Activity of SLP-L3.0</u>

SLP-L3.0 was purified from E. coli strain pPT0278 using standard extraction and protein separation techniques. Purity of the final product was determined by amino acid compositional analysis and microchemical elemental analysis to be 94.6% by weight.

SLP-L3.0 was evaluated for its ability to promote the attachment of a fibrosarcoma cell line, HT1080, commonly used for attachment assays on collagen, fibronectin and laminin. The purified product was dissolved in a solution of 4.5 M LiClO4 and diluted in phosphate buffered saline (PBS) to concentrations ranging from 100 to 0.14 µg/ml. 0.1 ml of the diluted polymer solution was dispensed to individual wells of a tissue culture polystyrene multi-well dish. The solution was left in contact with the surface of the dish for 2 hours then the dish was rinsed with PBS several times and incubated with freshly harvested HT1080 cells in serum-free medium. After one hour, unattached cells were removed by rinsing in PBS and attached cells were fixed and stained with a blue dye. The stained cells were quantified by solubilizing the dye and determining its solution absorbance by spectrometry at a wavelength of 595 nm.

The attachment experiment was run in parallel with wells coated with fibronectin and a synthetic peptide of the sequence RKQAASIKVAVS. Figure 1 shows a titration curve for cell attachment as a function of coating concentration. The results indicate that SLP-L3.0 (shown as LAM 3.0) promotes the attachment of HT1080 cells to tissue culture polystyrene coated with the polymer in a dose dependent manner. Maximum activity is observed at the greatest coating concentration used in this experiment, 100 µg/ml. Although the activity drops with coating concentration, cell attachment is observed greater than background even at the lowest concentration, 0.14 µg/ml. The polymer has significantly greater activity than the laminin peptide at concentrations of 74 µg/ml or less. Considering the difference in molecular weight

between the polymer and the synthetic peptide, the polymer has 32 times greater activity than the peptide on the basis of number of active sequences. The polymer compares favorably with the attachment activity of fibronectin, even though different binding receptors would be utilized in each case.

In order to evaluate the polymer's ability to stimulate neurite outgrowth, PC12 cells were grown on plastic dishes coated with polylysine, collagen type I, fibronectin, the peptide RKQAASIKVAVS, SLP-L3.0, and laminin. The cells were stimulated with nerve growth factor to undergo neural differentiation. The number and lengths of neural cell processes that extended from these cells were observed. SLP-L3.0 did promote neural outgrowth of PC12 cells to a significantly greater degree than uncoated dishes. The activity of SLP-L3.0 was greater than all of the substrates except laminin.

Extrusion of polystyrene coated with ProNectin®F

Crystalline polystyrene (PS) pellets (454 grams) were washed with isopropyl alcohol (500 ml per wash) three times to remove surface contaminants and then air dried. The pellets were rinsed in 800 mls of phosphate buffered saline (PBS). A solution containing 200 mg of ProNectin®F (SLPF batch RX4, Protein Polymer, Technologies, Inc.) dissolved in 20 mls of 4.5 M lithium perchlorate was diluted by adding 780 ml of PBS to yield a final ProNectin®F concentration of 0.25 mg/ml. The solution was then added to the pellets and stirred gently overnight for maximum adsorption of the ProNectin®F on the polystyrene surface. The pellets were then rinsed three times with deionized water then air dried.

The amount of ProNectin®F adsorbed to the surface of the polystyrene pellets was measured by amino acid analysis. 253 mg of coated pellets were placed in a sealed glass hydrolysis vial containing 100 µl of 0.1 N HCl and flushed with nitrogen gas. The vial was incubated at 100°C for 24 hrs. The liquid contained in the vial was removed and the pellets rinsed with 150 µl of 0.1 N HCl. The hydrolysis solution and the rinse were combined in a tube and dried under vacuum. The amino acid residues contained in the tube were derivatized by addition of isophenylthiocyanate according to standard protocols. The derivatized amino acids were separated by HPLC reverse phase chromatography, detected spectroscopically and quantified based on their adsorption as compared to known standards. The pellet hydrolysis was shown to contain 6.6 µg of amino acids per 253 mg of coated polystyrene or 26.1 µg of amino acids/g polystyrene. The dimensions of an average pellet were measured and the surface area was calculated to be approximately 0.3 cm^2/13 mg or 23 cm^2/g. Therefore, the coated pellets contained on average 26.1 µg of amino acids/23 cm^2 of polystyrene or 1.1 ug/cm^2. That ProNectin®F was the only source of the amino acids on the coated pellets is

evidenced by the fact that the amino acid content of the polystyrene hydrolysate closely matched the amino acid content of the ProNectin®F batch used in the coating. Accordingly, this procedure deposited approximately 1.1 μg of ProNectin®F per cm^2 of polystyrene surface area.

The dried ProNectin®F-coated pellets were fed into a heated single screw extruder fitted with a 2" x 1/8" ribbon die. Temperature was controlled in the extruder barrel to maintain the polystyrene melt temperature at 390° Fahrenheit. A first extrusion was made on a moving belt to produce a continuous ribbon. The ribbon was allowed to cool and then cut into 2" x 2" pieces. A second extrusion was conducted whereby the continuous ribbon was passed to a Carver press fitted with heated platens and compressed to 1/16" thickness. The cooled ribbon was then cut into 2" x 2" pieces.

Cut pieces of both the raw extruded and compression molded polystyrene ribbon were placed in 100 mm diameter polystyrene petri dishes. Delbecco's Modified Eagles Medium (DMEM) (25 ml) was added to the dishes to cover the cut pieces. Medium was also placed in empty polystyrene petri dishes to serve as negative controls. A suspension (25 ml) of viable African Green Monkey Kidney (VERO) cells at 4×10^5 were pipetted into all petri dishes and allowed to settle on the cut pieces or empty dish bottom. The petri dishes were then incubated for 1.5 hrs at 37° C in a 5% CO_2 atmosphere to allow cell attachment. The cut pieces and empty dishes were washed twice with PBS to remove unattached cells then 3% formaldehyde solution was added to fix attached cells by standing at room temperature for 5 min. The fixed cells were stained by adding 0.01% amido black in 40% methanol and 10% acetic acid solution. After 10 min staining the cut pieces and empty dishes were destained in 90% methanol, 8% acetic acid, 2% water. Attached cells were observed microscopically and photographed.

Both the raw extruded and compression molded polystyrene pieces showed considerable darkening on their surface indicating adsorption of the amido black stain by attached cells, whereas the empty polystyrene dishes showed no color development on the dish bottom indicating the lack of attached cells. Microscopic observation confirmed that the raw extruded and compression molded polystyrene surfaces were covered with attached cells, whereas the empty polystyrene dishes showed no color development on the dish bottom indicating the lack of attached cells. Microscopic observation confirmed that the raw extruded and compression molded polystyrene surfaces were covered with attached cells, whereas the empty polystyrene dishes lacked cell attachment.

The presence of attached cells on the extruded polystyrene and lack of attached cells on the empty dishes substantiates that the functionality of ProNectin®F as a cell attachment ligand was maintained through the extrusion process.

Non-Washed Powdered Polystyrene

A mixture of solid dry ice for cooling and polystyrene pellets without added zinc stearate, lubricants, or waxes (Amoco IR3-C0) was ground to a powder without added suspending liquid using a standard laboratory Waring blender. The polystyrene powders were sized using American Standard stainless steel sieves. Polystyrene powder of >100 mesh was taken for these experiments. No attempt was made to wash the polystyrene after the grinding operation. ProNectin®F (4 mg) was dissolved in 30 ml of 85% formic acid, slurried with 10 g of the sieved polystyrene powder, and concentrated to dryness on the rotary evaporator using bath temperatures of less than 60° C. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene powder between 304 stainless steel plattens using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 Kg for 5-10 seconds. Disks of 7 mm diameter were punched out of the films and were placed in individual wells of a 96 well tissue culture plate. The cell attachment assay was conducted as described above. Film samples were assayed in quadruplicate. The goal of this set of experiments was to explore methods of washing the films after they were installed into the tissue culture plate. Combinations of isopropanol, 1 mM aqueous EDTA, and 1% (w/v) Triton X-100 were used for these washes. A few disks in two lanes showed positive cell attachment signals. No obvious correlation with processing protocols could be established from these experiments.

Attachment Assay Using VERO Cells

The silicone grease "adhesive" used to affix compression molded test disks to the tissue culture plates was prepared by diluting Dow Corning High Vacuum Grease with cyclohexane to a final concentration of 25% w/v, centrifuging to compact the silica filler, and retaining the supernatant. To each well of a 96-well tissue culture plate which is to receive a test disk was added 25 μl of the silicone adhesive solution. The plate was then dried overnight in a vacuum oven at 40° C. In all operations, best results were obtained when all wash and aspiration steps were performed using a Biotek 403H automated plate washer with settings: dispense height = 10; plate height = 100; dispense volume = 200 μl; and number of washes = 2.

One lane of the plate, as a positive control, was solution coated with ProNectin®F. A stock solution of ProNectin®F (1 mg/ml) was prepared in 4.5 M

aqueous lithium perchlorate. This stock solution (10 μl) was diluted into 10 ml of 1X calcium-magnesium free phosphate buffered saline (cmf PBS) to yield a coating solution with a final concentration of 1 μg/ml. Coating solution (100 μl) was added to each well which is to serve as a positive control and incubated at room ambient temperature for 1.5 hours.

While the positive control lane was being solution coating with ProNectin®F, the 7 mm diameter test disks were placed into 5 dram vials and washed 3 times with 5 ml of 1X cmf PBS. The test disks were then placed into each well and gently pressed into the silicone grease which had been layered on the bottom of the well. After mounting the test disks into the plate, they were washed twice with 200 μl of 1X cmf PBS. At this time, the background absorbances were read using a Titertek Plate Reader at 595nm. Blocking solution (2mg of bovine serum albumin per milliliter of cmf PBS), 100 μl, was added to each well and incubated for 2 hours at room ambient temperature and one hour at 37°C. The blocking solution was aspirated and the plate rinsed once with 1X cmf PBS.

VERO cells, 2 X 10^5 cells from a suspension prepared at 2 x 10^6 cells/ ml, were added to each well using DME culture media without fetal bovine serum and the plate incubated for 1 hour in 5% carbon dioxide at 37° C. The media containing the cells was aspirated and the plate rinsed twice with 200 μl 1X cmf PBS. Fixative solution (3.7% formaldehyde in 1X cmf PBS), 100 μl, was added to each well and incubated for 5-10 minutes at room ambient temperature. The fixative solution was aspirated. Staining solution (0.1% amido black in 40% methanol-10% acetic acid), 100 μl, was added, and the plate was incubated for 30-60 minutes at room ambient temperature. The plate was then aspirated and rinsed with deionized water to remove all soluble dye. Absorbances were read using a Titertek Plate Reader at 595 nm.

Effects of Additives In the Polystyrene

Experiments were conducted using polystyrene pellets from a lot without added zinc stearate, mineral oil, or wax. The native pellets were reduced to a powder by dry grinding in a coffee grinder. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to various mesh sizes. The ProNectin®F was deposited onto the polystyrene powder of 60-80 mesh using a modified vortex dilution technique. The polymer powders (5.0g) were slurried in 15 ml of water. While vortexing, a solution of ProNectin®F in formic acid (1mg/15ml) was added in one portion. While vortexing, water (30ml) was added over about 60 seconds.

Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press

with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch". All cell attachment assays were conducted on a single 96-well plate according to our standard protocols.

Table 3.

Optical Densities of Cell Attachment Assays on Compression Molded Films with PnF.

Sample	PnF [ppm]	Polymer Disks	N	OD [mean]	±s	CV
Bare Plate			8	0.000	±0.003	n/a
Bare Plate	Solution coated 1μg-PnF/ml		8	0.797	±0.037	5%
102,028-1	PS[IR3-C0] Solution coated 1μg-PnF/ml		8	0.669	±0.070	10%
102,028-9	PS[GR3-C7] Solution coated 1μg-PnF/ml		8	0.681	±0.074	11%
102,028-1	000	PS [Amoco IR3-C0]	8	0.000	±0.010	n/a
102,028-9	000	PS [Amoco GR3-C7]	8	0.000	±0.028	n/a
102,028-2	200	PS [Amoco IR3-C0]	8	0.557	±0.046	8%
102,028-10	200	PS [Amoco GR3-C7]	8	0.106	±0.117	111%

In the above table, the polystyrene sample [Amoco IR3-C0] has no added zinc stearate or added waxes, while the polystyrene sample [Amoco GR3-C7] has 1700 ppm zinc stearate and added waxes.

After the disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities were measured. The optical densities reported in the above table are corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus becomes its own control. The optical densities are also corrected for the fact that the disks tend to pick up a little color during the staining process with the amidoblack chromophore. The cell attachment experiment in this case was the standard assay for fibronectin activity using VERO cells. In order to further validate this particular assay, one bare lane was coated with ProNectin®F directly onto the polystyrene plate using standard solution coating

methods. This acts as a check on the "temperament" of the particular batch of VERO cells used to conduct the assay.

The performance of ProNectin®F which was coated from solution onto polystyrene surfaces is unaffected by the presence or absence of processing aids added to the underlying polystyrene by the manufacturer. On the other hand, the performance of ProNectin®F which was dispersed into polystyrene powder and compression molded into films was seriously reduced by the presence of such processing aids. In the case of Amoco GR3-C7, the most likely cause of the degraded performance is believed to be the zinc stearate added as a mold release agent.

ProNectin®F, SLP3, and P-85 Surfactant

Polystyrene pellets without added zinc stearate, lubricants, or waxes (Amoco IR3-C0) was ground to a powder in a standard laboratory Waring blender using neat isopropanol as the slurrying agent. The recovered powder was further washed with isopropanol on a Buchner funnels, dried in air, and sieved to >100 mesh using American Standard stainless steel sieves. The additives used in these experiments were the protein polymers ProNectin®F and SLP3. The surfactant used was Pluronic P-85 from BASF Corporation. The protein polymers SLP3 and SLPF share a common backbone with the exception that SLPF includes a cell binding domain. Thus SLP3 serves as a negative control for the performance of the SLPF. The various additives were dissolved in 30 ml of formic acid (85%), slurried with 10 g of sieved polystyrene powder >100 mesh, and concentrated to dryness on a rotary evaporator. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150°C and a maximum force of 1500 Kg for 3-5 seconds. The total time for the molding cycle was about 15 seconds. Four sample disks for cell attachment assay were taken from the center of each film using a standard 7mm "one-hole" paper punch. All cell attachment assays were conducted on a single 96-well tissue culture plate using the cell attachment assay protocols described above.

Table 4. Cell Attachment Assays

	PnF [ppm]	P-85 [ppm]	SLP3 [ppm]	N	OD [mean]	±s	CV
102,017-00	Solution coated with 100 μl of 1μg-PnF/ml			8	0.608	±0.159	26%
102,017-01	-0-	-0-	400	6	0.122	±0.034	28%
102,017-02	-0-	400	400	6	0.116	±0.020	17%
102,017-03	400	-0-	-0-	8	0.642	±0.079	12%
102,017-04	50	100	-0-	8	0.166	±0.033	20%
102,017-05	800	100	-0-	8	0.513	±0.144	28%
102,017-06	200	200	-0-	8	0.116	±0.027	24%
102,017-07	-0-	400	-0-	8	0.091	±0.020	22%
102,017-08	100	400	-0-	8	0.100	±0.017	17%
102,017-09	400	400	-0-	8	0.101	±0.017	17%
102,017-10	200	800	-0-	8	0.095	±0.015	16%
102,017-11	50	1600	-0-	6	0.115	±0.033	28%
102,017-12	800	1600	-0-	6	0.178	±0.048	27%

Optical densities were measured at 595 nm, which is to say that attached cells stain with a blue color. There exists a modest background optical absorbance in these measurements due to a slight opacity of the inserted disks as well as the presence of the silicone grease used as adhesive. The measured optical densities of the 102,017-02 and -03 samples serve as a measure of this background absorbance. As can be seen in the data in the above table, the performance of the disks from sample 102,017-03 is indistinguishable from that of Polystyrene disks which were coated with ProNectin®F from solution in aqueous lithium perchlorate. Only low levels of the P-85 surfactant can be tolerated in combination with the ProNectin®F without seriously degrading the cell attachment performance of the ProNectin®F. Again, these results demonstrate the sensitivity of the cell attachment performance of the ProNectin®F to interferences from additives present in the polystyrene.

Deposition of ProNectin®F onto Polystyrene by Evaporative Coating

Experiments were conducted using Polystyrene pellets (Amoco IR3-C0) from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder using neat isopropanol as the slurrying agent in a miniature Waring blender. The recovered powder was further washed with isopropanol on a Buchner funnel, air

dried, and sieved to various mesh sizes. The ProNectin®F was dissolved in 30 ml of formic acid, slurried with 10 g of sieved polystyrene powder-, and concentrated to dryness on a rotary evaporator. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch." All cell attachment assays was conducted on a single 96-well plate using the standard protocol described above.

Table 5.

Optical Densities of Cell Attachment Assays On Compression Molded PS With PnF

	PnF [ppm]	PS Powder mesh range	N	OD [mean]	±s	CV
Bare Plate	Optical blank, no cells		8	0.037	±0.002	4%
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml		8	0.573	±0.024	4%
103-64-00	PS Optical & Stain blank; no cells		8	0.085	±0.035	41%
103-64-00	PS solution coated with 100 µl 1 µg/-PnF/ml		8	0.600	±0.075	13%
103-64-J	150	20-40	8	0.500	±0.061	12%
102-17-03	400	>100	8	0.493	±0.069	14%
103-64-A	400	>100	8	0.461	±0.045	10%
103-64-B	200	>100	8	0.352	±0.086	24%
103-64-C	150	>100	8	0.181	±0.055	30%
103-64-F	150	60-80	8	0.471	±0.097	20%
103-64-G	150	20-40	8	0.353	±0.074	21%
103-64-D	100	>100	8	0.115	±0.056	49%
103-64-E	50	>100	8	0.012	±0.049	408 %

After the polystyrene disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities were measured. The optical densities reported in the above table are corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus becomes its own control. The optical densities are also corrected for the fact that

the polystyrene disks tend to pick up a little color during the staining process with the amidoblack.

Data from samples prepared with >100 mesh powder is especially well behaved with cell attachment performance decreasing monotonically with the concentration of applied ProNectin®F.

At 400 ppm PnF compression molded into this polystyrene powder, the performance of the disks is statistically indistinguishable from PnF at 1μg/ml solution coated onto the same disks or onto the bare plate. Below 250-300 ppm, the performance begins to fall off.

Strangely enough, the above data shows that PnF at 150 ppm on 60-80 mesh polystyrene powder performs as well as PnF at 400 ppm on >100 mesh. Thus the mesh size of the polystyrene powder appeared to be a determinant of the performance when ProNectin®F was deposited onto the polystyrene by evaporative coating.

Deposition of ProNectin®F onto Polyethylene by Evaporative Deposition

Experiments were conducted using low density polyethylene (PE) powder commercially available from Aldrich Chemical Company as catalog number 18,189-7. The powder is of unknown composition with respect to processing aids or stabilizers added by the manufacturer. In order to reduce the possibility of interferences by these additives, the PE powder was subjected to exhaustive extraction with boiling isopropanol for 24 hours in a Soxhlet apparatus. Recovered polyethylene powder was air dried before use. The ProNectin®F was dissolved in 30 ml of formic acid, slurried with 10 g of PE powder, and concentrated to dryness on a rotary evaporator. Several sample films from each lot of powder were compression molded from 250 mg of coated polyethylene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch". All cell attachment assays was conducted on a single 96-well plate.

Table 6.

Optical Densities of Cell Attachment Assays On Compression Molded PE With PnF.

	ProNectin®F On Polyethelene Powder	N	OD [mean]	±s	CV
Bare Plate	Optical blank	8	0.032	±0.002	6%
Bare Plate	Solution coated with 100 μl of 1 μg-PnF/ml	8	0.721	±0.022	3%
103,064-00	Solution coated with 100 μl of 1 μg-PnF/ml	8	0.591	±0.052	9%
103,064-01	600 ppm	8	0.092	±0.060	65%
103,064-02	400 ppm	8	0.085	±0.061	72%
103,064-03	200 ppm	8	0.169	±0.100	59%
103,064-04	150 ppm	8	0.138	±0.084	75%
103,064-05	100 ppm	8	0.084	±0.063	75%
103,064-06	50 ppm	8	0.066	±0.067	102%
103,064-00	00 ppm	8	0.000	±0.036	n/a

After the disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities was measured. The optical densities reported in the above table are corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polyethylene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus becomes its own control. The optical densities are also corrected for the fact that the disks tend to pick up a little color during the staining process iwht the amidoblack dyestuff. In order to further validate this particular assay, one bare lane was coated with ProNectin®F directly onto the polystyrene plate using standard solution coating methods.

Clearly, ProNectin®F which was evaporatively coated onto polyethylene powder and compression molded into films is active in a cell attachment assay using VERO cells. The degree of attachment activity is lower compared to that observed on polystyrene. The difference in performance between polystyrene and polyethylene have been due to additives in the polyethylene which were not removed in the washing step.

Deposition of ProNectin®F onto Polypropylene and Polymethylmethacrylate by Vortex Dilution.

Polypropylene non-woven fabric was recovered from the liner of a disposable diaper and washed exhaustively with isopropanol in Soxhlet apparatus for 24 hours.

This fabric represents a conveniently available source polypropylene with a high surface area suitable for coating with ProNectin®F. Coating was conducted using the modified vortex dilution technique as described in the section entitled "Effects of Additives in the Polystyrene". Polymethylmethacrylate (Aldrich Chemical Company catalog number 18,224-9) as a medium molecular weight powder was used without additional purification. Coating was conducted using the modified vortex dilution technique.

Several sample films from each lot of coated polymer were compression molded from 250 mg samples between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150°C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7mm "one-hole-punch". All cell attachment assay was conducted on a single 96-well plate using the standard protocols described above.

Table 7.

Deposition of ProNectin®F onto Polypropylene and Polymethylmethacrylate.

Sample	PnF [ppm]	Polymer Disks	N	OD [mean]	±s	CV
Bare Plate			8	0.000	±0.003	n/a
Bare Plate	Solution coated 1μg-PnF/ml		8	0.797	±0.797	5%
102,028-1	000	PS [Amoco IR3-C0]	8	0.000	±0.010	n/1
102,028-2	200	PS [Amoco IR3-C0]	8	0.557	±0.046	8%
102,028-7	000	Poly[Methyl Methacrylate]	8	0.000	±0.038	n/1
102,028-8	200	Poly[Methyl Methacrylate]	8	0.435	±0.050	11%
102,028-11	000	Polypropylene	8	0.000	±0.042	n/a
102,028-12	230	Polypropylene	8	0.252	±0.128	51%

These results showed that ProNectin®F can be compression molded into polypropylene and polymethylmethacrylated films. Neither the polypropylene nor the polymethylmethacrylate was of known composition with respect to low level

concentrations of additives and surfactants. These particular samples of polymers were used because they were readily available and not because they were known to be optimal for this applicaiotn. In any case, these samples of polymers showed cell attachment activity when compression molded with ProNectin®F.

<u>Effect of Deposition Methods and Mesh Size</u>

Experiments were conducted using polystyrene pellets (Amoco IR3-C0) from a lot without added zinc stearate, mineral oil, or wax. The native pellets were reduced to a powder by dry grinding in a coffee grinder. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to various mesh sizes. The ProNectin®F was deposited onto the polystyrene powders using one of three methods: evaporation of the formic acid in the absence of water, referred to as "Dry" rotovap; evaporation of the formic acid after the addition of 5 ml of water, referred to as "Wet" rotovap; and dilution of the formic acid with water under vortexing conditions, referred to as "Vortex" dilution. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch". All cell attachment assay was conducted on a single 96-well plate using the standard protocol described above.

Table 8.
Optical Densities of Cell Attachment Assays On Compression Molded PS With PnF.

Sample	PnF [ppm]	Deposition Method	PS Powder mesh range	N	OD [mean]	±s	CV
103,065-00	Optical blank, cells & stain			8	0.000	±0.032	n/a
103,065-00	Solution coated with 100 µl of 1 µg-PnF/ml			8	0.655	±0.099	15%
103,065-A	150	Dry Rotovap	>100	8	0.112	±0.049	44%
103,065-B	150	Wet Rotovap	>100	8	-0.008	±0.051	n/a
103,065-C	150	Vortex Dil'n	>100	8	0.468	±0.112	24%
103,065-D	150	Dry Rotovap	60-80	8	0.151	±0.045	30%
103,065-E	150	Wet Rotovap	60-80	8	0.335	±0.083	25%
103,065-F	150	Vortex Dil'n	60-80	8	0.532	±0.071	13%
103,065-G	150	Dry Rotovap	20-40	8	0.239	±0.044	18%
103,065-H	150	Wet Rotovap	20-40	8	0.102	±0.081	79%
103,065-I	150	Vortex Dil'n	20-40	8	0.410	±0.043	10%
103,065-J	150	Wet Rotovap	40-60	8	0.149	± 0.72	408%

After the disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities was measured. The optical densities reported in the above table were corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus becomes its own control. The optical densities are also corrected for the fact that the disks tend to pick up a little color during the staining process with the amidoblack. In order to further validate this particular assay, one bare lane was coated with ProNectin®F directly onto the polystyrene plate using standard solution coating methods. This acts as a check on the "temperment" of the particular batch of cells used to conduct the assay.

The data in Table 8 showed that the most efficient utilization of ProNectin®F coated onto polystyrene powders comes about using the vortex dilution method. The mesh size of the polystyrene powder did not seem to be a major determinant of the outcome of the vortex dilution coating process. The use of mixed mesh polystyrene powders eased the preparation of these powders because the useable fraction ground powder increases with the broader mesh range.

Water present during evaporative deposition has an effect on the outcome of the coating process. This observation could explain variablility observed in some of the

evaporative coating experiments. Variable amounts of water could enter the rotary evaporator from room ambient water vapor through accidental leaks or through sparging of the rotary evaporator apparatus with air during the evaporation process.

Deposition of ProNectin®F onto Polystyrene by Vortex Dilution Coating

Experiments were conducted using polystyrene (PS) pellets (Amoco IR3-C0) from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to >20 mesh. The ProNectin®F was dissolved in 15 ml of formic acid, slurried with 5 g of sieved polystyrene powder, agitated on a vortex mixer, and diluted with 45 ml of deionized water. The recovered polystyrene powder was collected on a Buchner funnel and dried in air. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150°C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7mm "one-hole-punch." All cell attachment assay was conducted on a single 96-well plate using the standard protocol described above.

Table 9.

Optical Densities of Cell Attachment Assays On Compression Molded PS With PnF

	PnF [ppm]	PS Powder mesh range	N	OD [mean]	±s	CV
Bare Plate	Optical blank, no cells or stain		8	0.037	±0.001	3%
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml		8	0.799	±0.026	3%
103-66-00	PS Optical blank, no cells or stain		8	0.007	±0.016	229%
103-66-00	PS blank, cells and stain		8	0.022	±0.012	55%
103-66-00	Solution coated with 100 µl of 1 µg/-PnF/ml		8	0.747	±0.052	7%
103-66-A	300	>20	8	0.516	±0.048	9%
103-66-B	250	>20	8	0.641	±0.073	11%
103-66-C	200	>20	8	0.627	±0.068	11%
103-66-D	150	>20	8	0.650	±0.057	9%
103-66-E	100	>20	8	0.544	±0.067	12%
103-66-F	50	>20	8	0.592	±0.060	10%

After the polystyrene disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities was measured. The optical densities reported in the above table were corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus became its own control. The optical densities are also corrected for the fact that the polystyrene disks tend to pick up a little color during the staining process with the amidoblack.

ProNectin®F retained its activity down to 50 ppm. The combination of mixed mesh powders with vortex dilution deposition is extremely efficacious in depositing PnF in active form.

The reason for the reduced effectiveness of evaporative deposition compared to vortex deposition may be hydrolysis of the protein. The efficacy of vortex deposition bodes very well for the economics of the process.

Compression Molding of Polystyrene Containing ProNectin L

Experiments were conducted using polystyrene pellets from a lot without added zinc stearate, mineral oil, or wax. The native pellets were reduced to a powder by dry grinding in a coffee grinder. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to various mesh sizes. The ProNectin®L (PnL or SLPL 3.0) was deposited onto the polystyrene powder of 60-80 mesh using the vortex dilution technique. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch". A cell attachment assay was conducted on a single 96-well plate as described below.

Table 10.

Optical Densities of Cell Attachment Assays On Compression Molded PS With ProNectin®L.

	PnL [ppm]	Deposition Method	PS Powder mesh range	N	OD [mean]	±s	CV
Bare plate	Optical blank, cells & stain			8	0.000	±0.014	n/a
Bare plate	Solution coated with 100 µl of 1 µg-PnF/ml			8	0.767	±0.106	14%
103,067-00	PS optical blank, cells & stain			6	0.000	±0.016	n/a
103,067-00	PS, solution coated with 100 µl of 1 µg-PnF/ml			8	0.797	±0.144	18%
103,065-A	500	Vortex	60-80	8	0.052	±0.038	73%
103,065-B	550	Vortex	60-80	8	0.043	±0.043	100%
103,065-C	150	Vortex	60-80	8	0.046	±0.031	67%
103,065-C1	150	Vortex	60-80	6	0.342	±0.106	31%

After the disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities were measured. The optical densities reported in the above table were corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus becomes its own control. The optical densities are also corrected for the fact that the disks tend to pick up a little color during the staining process with the amidoblack. The cell attachment experiment in this case was the standard assay for laminin activity using RD P56 cells, described below. In order to further validate this particular assay, one bare lane was coated with ProNectin®L directly onto the polystyrene plate using standard solution coating methods. This acts as a check on the "temperment" of the particular batch of RD-P56 cells used to conduct the assay.

This data shows that ProNectin L can survive the thermal history of the compression molding process. After drying in air, the vortex dilution coated polystyrene powders retained a slight odor of formic acid. The sample 103,067-C1 was compression molded as it stood. The other samples were sparged with a stream of dry nitrogen until all odor of formic acid was removed. It could be that the IKVAV sequences of the ProNectin L in sample 103,067-C1 were stabilized towards thermal degradation by neutralization of the lysines with this residual formic acid. In particular, more reproducible results are obtained when non-volatile acids such as toluene sulfonic acid are added to the compression molding mixtures.

Attachment Assay Using RD-P56 Cells

The silicone grease "adhesive" used to affix compression molded test disks to the tissue culture plates was prepared by diluting Dow Cornin High Vacuum Grease with cyclohexane to a final concentration of 25% w/v, centrifuging to compact the silica filler, and retaining the supernatant. To each well of a 96-well tissue culture plate which is to receive a test disk was added 25 µl of the silicone adhesive solution. The plate was then dried overnight in a vacuum oven at 40°C. One lane of the plate, as a positive control, was solution coated with ProNectin®L using the standard protocols described above. Best results were obtained when all wash and aspiration steps were performed using a Biotek 403H automated plate washer with settings: dispense height = 10; plate height = 100; dispense volume = 200 µl; and number of washes = 2.

While the positive control lane was being solution coating with ProNectin®L, the 7 mm diameter test disks were placed into 5 dram vials and washed 3 times with 5 ml of 1X calcium-magnesium free phosphate buffered saline (cmf PBS). The test disks were then placed into each well and gently pressed into the siicone grease which had been layered on the bottom of the well. After mounting the test disks into the plate, they were washed twice with 100 µl of 1X cmf PBS. At this time, the background absorbances were read using a Titertek Plate Reader at 595 nm. Blocking solution (2mg of bovine serum albumin per milliliter of cmf PBS), 100 µl, was added to each well and incubated for 30 minutes at 37° C. The blocking solution was aspirated and the plate rinsed once with cmf PBS. RD-P56 cells, 1 X 10^5 cells, were added to each well using culture media without or fetal bovine serum and the plate incubated for 1 hour 37° C. The media containing the cells was aspirated and the plate rinsed twice with cmf PBS. Fixative solution (3.7% formaldehyde in 1X cmf PBS) was added to each well and incubated for 20 minutes at room ambient temperature. The fixative solution was aspirated and the plate was rinsed once with cmf PBS. Staining solution (0.1% amido black in 40% methanol-10% acetic acid) was added, and the plate was incubated for 15 minutes at room ambient temperature. The plate was then rinsed with deionized water to remove all soluble dye. The plate was dried over night at room ambient temperature. Elution buffer (10% aqueous sodium dodecyl sulfate) was added and the absorbance read using the Titertek Plate Reader at 595 nm.

Reproducibility of Coatings Deposited by Vortex Dilution

Experiments were conducted using polystyrene pellets (Amoco IR3-C0) from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to

>20 mesh. The ProNectin®F was dissolved in 15 ml of formic acid, slurried with 5 g of sieved polystyrene powder, agitated on a vortex mixer, and diluted with 45 ml of deionized water. The recovered polystyrene powder was collected on a Buchner funnel and dried in air. Three independent preparations of polystyrene powder coated with ProNectin®F were conducted and sampled individually for compression molding. The three preparations were combined, physically mixed, and again sampled for compression molding.

Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch". A cell attachment assay was conducted on a single 96-well plate using the standard protocol described above.

Table 11.

Optical Densities of Cell Attachment Assays on Compression Molded PS with PnF.

	PnF [ppm]	PS Powder mesh range	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 μl of 1 μg/-PnF/ml		8	0.547	±0.019	3%
103-069-00	PS Optical blank, no cells or stain		7	0.000	±0.024	n/a
103-069-00	PS blank, cells and stain		8	0.555	±0.036	6%
103-069-A	200	>20	8	0.473	±0.031	9%
103-069-B	200	>20	8	0.526	±0.046	7%
103-069-C	200	>20	8	0.521	±0.071	14%
103-069-D	200	>20	7	0.535	±0.033	6%

The data in Table 11 showed that multiple preparations of a coated polystyrene powder gave the same result. The coating process appeared reproducible. The mean optical density for preparations A, B, C & D, which is [A+B+C], was 0.520 ± 0.047. The results in Table 11 showed that the application of ProNectin®F to polystyrene powder can be accomplished with high reproducibility on a batch-to-batch basis. Establishing such reproducibility is important to the design of coating processes at a commercial scale.

<u>Usable Dispersing Agents</u>

Experiments were conducted using polystyrene pellets (Amoco IR3-C0) from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a

powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to >20 mesh. The ProNectin®F was dissolved in 15 ml of formic acid, slurried with 5 g of sieved polystyrene powder, agitated on a vortex mixer, and diluted with 45 ml of deionized water. The recovered polystyrene powder was collected on a Buchner funnel and dried in air.

Polystyrene powder which was precoated with ProNectin®F was then challenged with a wash with isopropanol alone and in combination with reagents which are potentially useful as agents for the dispersal of ProNectin®F into polystyrene melts. The reagents, each at 200 ppm in isopropanol (103,069-E), were phenyltriethoxysilane (103,069-F), tetraisopropoxytitanium (IV) (103,069-G), isopropoxytris isosteroyl titanate (Kenrich KR-TTS) (103,069-I), neopentyl (diallyl) oxy-[trix(dioctyl)pyrophosphato] titanate (Kenrich LICA-38) (103,069-H).

Preparation of compression molded film samples and a cell attachment assay were conducted using standard protocols described above.

Table 12.

Optical Densities of Cell Attachment Assays on Compression Molded PS with PnF.

	PnF [ppm]	PS Powder mesh range	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml		8	0.547	±0.019	3%
103,069-00	PS Optical blank, no cells or stain		7	0.000	±0.024	n/a
103,069-00	PS blank, cells and stain		8	0.555	±0.036	6%
103,069-A	200		8	0.473	±0.031	9%
103,069-E	200		8	0.482	±0.073	15%
103,069-F	200		7	0.512	±0.040	8%
103,069-G	200		8	0.509	±0.048	10%
103,069-H	200		7	0.490	±0.067	14%
103-069-I	200		8	0.510	±0.042	8%

The data in table 12 showed that these dispersing agents do not interfere with the cell attachment function of the ProNectin®F when they are added to the coated polystyrene powder at these concentrations. These four dispersing agents are thus may be considered as aids for achieving improved mixing of the ProNectin®F throughout the polystyrene melt during a thermolding process.

Thermal Stress Matrix for Unstabilized ProNectin®F

Experiments were conducted using Polystyrene pellets from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to >20 mesh. The ProNectin®F (1.0 mg)was dissolved in 15 ml of formic acid, slurried with 5 g of sieved polystyrene powder, agitated on a vortex mixer, and diluted with 45 ml of deionized water. The recovered polystyrene powder was collected on a Buchner funnel and dried in air without agitation. This powder was designated as having been coated at 200 ppm.

A sample (2.0 g) of polystyrene powder coated with ProNectin®F (200 ppm) was placed into a Pyrex glass tube (16mm X 100mm); sealed with a rubber septum cap, sparged with nitrogen, and heated in a thermostatted oil bath preheated to the required temperature for varying lengths of time. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch." A cell attachment assay was conducted on a single 96-well plateusing the protocol described above.

Table 13.

Optical Densities of Cell Attachment Assays.

	PnF [ppm]	Time [min]	Temp.[°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µlof 1 µg/-PnF/ml			8	0.628	±0.008	1%
103-075-1	200	1/4	150°	8	0.530	±0.099	19%
103-075-2	200	5	150°	8	0.488	±0.069	14%
103-075-3	200	80	150°	8	0.200	±0.106	53%
103-075-4	200	20	175°	8	0.083	±0.042	51%
103-075-5	200	10	200°	8	0.066	±0.036	55%
103-075-6	200	20	200°	8	0.036	±0.031	86%
103-075-7	200	40	200°	8	0.010	±0.020	200%
103-075-8	200	20	225°	8	0.008	±0.011	138%
103-075-9	200	5	250°	8	0.011	±0.014	127%
103,075-10	200	40	250°	8	0.0003	±0.011	n/a
103,075-11	200	10	275°	8	0.0003	±0.015	n/a

After the polystyrene disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities were measured. The optical densities reported in the above table are corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus becomes its own control. The optical densities are also corrected for the fact that the polystyrene disks tend to pick up a little color during the staining process with the amidoblack.

The performance of the compression molded disks of polystyrene in the cell attachment assay decreased with increasing time and with increasing temperature in a complex manner. The amount of ProNectin®F required in the initial formulation will be dictated by the required performance of the final article and by the time-temperature history of the injection molding process.

The time-temperature experiments represent a response surfacewhich can be fitted by multivariate regression analysis. A model was defined which assumes a first order reaction for thermal degradation with the rate constant being exponential in temperature. The fit of this model is R2 = 0.963, and the equation has the satisfying property of fitting only three adjustable parameters to the 11 data points. The following parameters can be added together to form an equation which can be used to interpolate points on the time-temperature response surface.

Parameter estimates for Ln[Ln(A0/A)] R2 = 0.963

Term	Coefficient	Std Error	t Ratio	Prob>$\lvert t \rvert$
Intercept	12.256021	1.49242	8.21	0.0000
Ln[t]	0.7049276	0.08461	8.33	0.0000
1/°K	-6429.016	672.61	-9.56	0.0000

Relationship of Solution Concentrations to Surface Deposition

Films were compression molded from polystyrene pellets (Amoco IR3-C0) and cut into strips 1cm X 2cm. In order to reduce the fluorescent background due to low level contaminants as much as possible, the films were extracted in concentrated hydrochloric acid for 18 hours at 80° C. The disks were rinsed in deionized water and solution coated with ProNectin®F using a solution of PnF (1 mg/ml) in 88% formic acid serially diluted out to the final concentrations with 1X phosphate buffered saline. Polystyrene films were left in contact with the diluted coating solutions for 2 hours on a nutator to provide agitation. The films were recovered, rinsed in deionized water, and air dried. Proteins on the surface of the each piece of film were hydrolysed by exposure to the vapors of constant boiling hydrochloric acid in an evacuated container for 18 hours at 80° C. The vials were opened and placed in a vacuum oven at 40° C for 2 hours to remove water and hydrogen chloride. The residue of hydrolysed amino acids was dissolved using 1 ml of 100 mM pH9 borate buffer. Fluorescence was developed by adding 1 ml of a solution of fluorescamine (0.1 mg/ml) in acetone. Fluorescence was read using a Turner filter fluorimeter (excitation 390 nm; emmission 475 nm). Fluorescent standards were prepared using synthetic mixtures of glycine, alanine, and serine in a molar ratio corresponding to the PnF. In all cases the amount of PnF (Concsolution X Vol.) available in the diluted solutions to each piece of polystyrene film was much greater than the amount of PnF (Concsurface X Area) which ended up actually absorbed to the surface. This ratio is indicated at each data point of the graph. The means and standard deviations for each data point by conducting multiple assays on 6 to 8 independent samples.

Films were compression molded from polystyrene pellets (Amoco IR3-C0). Disks (7mm dia.) were punched out using a "one-hole" paper punch. These disks were cleaned and coated with ProNectin®F as described above. The disks were mounted in a 96-well tissue culture plate using our standard methods. One lane of blank well in the same plate were solution coated with PnF. The cell attachment assay was conducted according to the standard protocol described above.

Table 14.
Surface Concentration vs. Solution Concentration.

PnF Solution (µg/ml)	Excess PnF Available	PnF Surface (µg/cm^2-Ps)		Cell Attachment Abs @ 595	
		Mean	±Std Dev	Mean	±Std Dev
100.00	802x	0.624	±0.157	0.59	±0.06
10.00	140x	0.479	±0.145	0.55	±0.04
1.00	48x	0.104	±0.087	n/d	n/d
0.20	12.0x	0.084	±0.085	0.52	±0.02
0.10	8.2x	0.061	±0.066	0.29	±0.05
0.02	3.2x	0.031	±0.062	0.00	n/d
0.00	n/a	0.000	±0.077		

The deposition of PnF onto polystyrene surfaces showed a sigmoidal profile of surface concentrations versus solution concentrations which is a characteristic of Langmuirian absorption processes.

At high solution concentrations, the amount deposited onto the surface of the polystyrene reaches a plateau. The measured surface concentrations in this plateau region were very close to our estimate of monolayer coverage based on a consideration of the geometry of the ProNectin®F molecule.

Cell attachment activity of the coated polystyrene was remarkably insensitive to coverage of the polystyrene by PnF. Cell attachment reaches a plateau at about monolayer coverage of the surface by PnF. Half of the cell attachment activity persists down to 0.1 monolayer on the specially cleaned polystyrene surfaces. Cell attachment activity did not persist to such low degrees of coverage when PnF was deposited onto a standard commerical grade polystyrene such as Amoco 1R3-C0. The data in Table 14 relating solution concentrations of ProNectin®F to surface deposition and cell attachment performance is essential to designing a commerical coating process.

Stabilization of ProNectin®F

Experiments were conducted using Polystyrene pellets from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to >20 mesh. The ProNectin®F (1.0 mg)was dissolved in 15 ml of formic acid, slurried with 5 g of sieved polystyrene powder, agitated on a vortex mixer, and diluted with 45 ml of deionized water. In another experiment, the ProNectin®F (1.0 mg) and calcium oxide

(1.0 mg) was dissolved in the formic acid. In both cases, the recovered polystyrene powder was collected on a Buchner funnel and dried in air without agitation. In one case, the polystyrene powder was compression molded before all residues of formic acid were evaporated. These powders were designated as being coated at 200 ppm ProNectin®F.

Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7mm "one-hole-punch." A cell attachment assay was conducted on a single 96-well plate using the standard protocol described above.

Table 15.
Optical Densities of Cell Attachment Assays Using PnF(200 ppm) On PS

	Comments	Time [min]	Temp. [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml			5	0.761	±0.005	1%
103,066-C	Std Vortex	1/4	150°	3	0.681	±0.059	9%
103,079-2	Std Vortex	5	150°	4	0.516	±0.055	11%
103,079-3	Formic Residues	1/4	150°	3	0.204	±0.088	43%
103,079-4	Ca^{+2}	1/4	150°	3	0.729	±0.040	5%

After the polystyrene disks were mounted on the tissue culture plate with silicone grease dissolved in cyclohexane at 25% w/v, a matrix of optical densities were measured. The optical densities reported in the above table were corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus became its own control. The optical densities were also corrected for the fact that the polystyrene disks tend to pick up a little color during the staining process with the amidoblack.

Occasionally, difficulties were encountered in the cell attachment assays; especially with reduced optical densities for the positive controls. Some of these difficulties might have been a result of contamination by cyclohexane. Cyclohexane is used to apply the silicone grease which serves as an adhesive to afix the polystyrene disks to the bottoms of the wells on the 96-well plate. Removal of the cyclohexane is best achieved using a vacuum oven.

The presence of formic acid residues on samples of polystyrene coated with ProNectin®F, leads to reduced O.D.'s upon compression molding compared to fully dried samples. This result is in direct contrast to the result described above in the case of ProNectin®L.

The presence of calcium formate in the coating recipe may have a protective effect on the ProNectin®F during the compression molding operation.

Lot Reproducibility & Rinses with Calcium Salts

Experiments were conducted using Polystyrene pellets from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to >20 mesh. Samples of ProNectin®F from three separates lots were evaluated for their cell attachment efficacy. The ProNectin®F (1.0 mg)was dissolved in 15 ml of formic acid, slurried with 5 g of sieved polystyrene powder, agitated on a vortex mixer, and diluted with 45 ml of deionized water. The polystyrene powder was recovered by filtration, sucked as dry as possible on the filter, and dried in air. In another experiment, the polystyrene powder was recovered by filtration, rinsed with water, sucked as dry as possible, and dried in air.

In another group of experiments, samples of a single lot of ProNectin®F (1.0 mg) were dissolved in three solvents: 85% formic acid, 6 M aqueous urea, and 4.5 M aqueous lithium perchlorate. Coating of the polystyrnen powder was conducted as described above. Samples were recovered by filtration, rinsed with 100 mMolar aqueous calcium chloride solutions, sucked as dry as possible on the filter, and dried in air.

Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch". A cell attachment assay was conducted on a single 96-well plate using the standard protocol described above.

Table 16.

Optical Densities of Cell Attachment Assay

	Comments	Time [min]	Temp [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml			8	0.491	±0.041	1%
103,080-C	Lot #26; Formic	1/4	150°	8	0.356	±0.079	27%
103,080-D	Lot #27; Formic	1/4	150°	7	0.384	±0.079	11%
103,066-C	Lot #24; Formic	1/4	150°	8	0.374	±0.065	112%
013,080-H	Lot #26; Formic; Water Rinse	1/4	150°	7	0.361	±0.132	32%
103,080-I	Lot #26; Formic; $CaCl_2$ Rinse	1/4	150°	8	0.494	±0.043	13%
103,080-J	Lot #26; Urea; $CaCl_2$ Rinse	1/4	150°	8	0.548	±0.036	22%
103,080-K	Lot #26; $LiClO_4$; $CaCl_2$ Rinse	1/4	150°	8	0.519	±0.059	15%

After the polystyrene disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities were measured. The optical densities reported in the above table were corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus became its own control. The optical densities were also corrected for the fact that the polystyrene disks tend to pick up a little color during the staining process with the amidoblack.

Samples of ProNectin®F from three separate fermentation lots are all functionally equivalent in the cell attachment assay after being coated onto polystyrene powders and compression molded into films.

These data show that solutions of ProNectin®F made up in 85% formic acid, 6 M aqueous urea, and 4.5 M aqueous lithium perchlorate were all functionally equivalent when used in the vortex dilution method of coating polystyrene powders.

The ability to substitute 6 M aqueous urea is of great significance to the design and cost of a large scale coating process for polystyrene powders. Avoiding corrosive reagents in the process means that the large scale process equipement can be made of less expensive materials of construction.

Calcium Stabilization of ProNectin®F

Experiments were conducted using polystyrene (PS) pellets from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to >20 mesh.

ProNectin®F (8.0 mg)was dissolved in 8 ml of formic acid and added to 400 ml of 150 mM NaCl solution contained in a 1000 ml Erlenmeyer flask fitted with a magnetic stirrer to yield a final concentration of 20 µg-PnF/ml. The polystyrene powders were prewetted with mixtures of methanol by immersing the PS in the methanol and applying house vacuum to the head space. Excess methanol was decanted from the wetted PS powders before the PS powders were added to the diluted solution of PnF. The slurry was stirred for 2 hours at room temperature before being filtered. One sample of powder was rinsed with water, the sample of PS powder was resuspended in 100 mMolar calcium chloride solution, which was adjusted to pH 7, stirred for 10 minutes, and filtered. Theses samples of PS powder were then resuspended in 10 mMolar calcium chloride solution, which was adjusted to pH 7, stirred for 10 minutes, filtered, sucked as dry as possible and dried in air. A sample (2.0 g) of polystyrene powder coated with ProNectin®F was placed into a Pyrex glass tube (16mm X 100mm); sealed with a rubber septum cap, sparged with nitrogen, and heated in a thermostatted oil bath preheated to 200°C for 5 minutes.

Table 17.
Optical Densities of Cell Attachment Assays

	Comments	Time [min]	Temp. [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml			8	0.549	±0.044	1%
103,081-1A	Wetted 100% MeOH	1/4	150°	8	0.531	±0.094	18%
103,081-1B	Wetted 100% MeOH	5	200°	8	0.135	±0.066	49%
103,081-2B	Wetted 100% MeOH; Ca^{+2}	5	200°	7	0.194	±0.052	27%
103,081-3A	Wetted 50% MeOH	1/4	150°	8	0.560	±0.062	11%
103,081-3B	Wetted 50% MeOH	5	200°	8	0.065	±0.073	112%
103,081-4A	Wetted 50% MeOH; Ca^{+2}	1/4	150°	8	0.554	±0.079	14%
103,081-4B	Wetted 50% MeOH; Ca^{+2}	5	200°	7	0.311	±0.099	32%
103,081-5A	Dry Powder	1/4	150°	8	0.519	±0.065	13%
103,081-5B	Dry Powder	5	200°	8	0.172	±0.037	22%
103,081-6A	Dry Powder; Ca^{+2}	1/4	150°	8	0.581	±0.050	9%
103,081-6B	Dry Powder; Ca^{+2}	5	200°	8	0.418	±0.064	15%

Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of

each of three films using a standard 7 mm "one-hole-punch". All cell attachment assays were conducted on a single 96-well plate using the standard protocol described above.

After the polystyrene disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities were measured. The optical densities reported in the above table were corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus became its own control. The optical densities were also corrected for the fact that the polystyrene disks tend to pick up a little color during the staining process with the amidoblack.

ProNectin®F may be stabilized towards thermally induced deactivation. Rinsing the coated PS powders with calcium chloride solution produce samples which retained much more activity than the standard samples after a thermal challenge of 5 minutes at 200° C.

The coating methodology used in preparing these samples is important because it can be scaled for working with larger lots of polystyrene powder. The engineer aspects of working with stirred slurries of powders is well understood and is scaleable to larger sizes in a straightforward manner. The "tool", the coating technique, now exists which makes it possible to work with multi-kilogram lots of polystyrene powders.

Prewetting the polystyrene with methanol was conducted in order to improve the contact between the aqueous solution of PnF and the hydrophobic surface of the polystyrene powders. Such a prewetting is clearly counterindicated by the data in this table. Coating of dry polystyrene powders performed better. The effect was more apparent in those samples which were "stressed" at high temperatures.

<u>Thermal Stress Matrix for Calcium Stabilized ProNectin®F</u>

Experiments were conducted using Polystyrene pellets from a lot without added zinc stearate, mineral oil, or wax. The native pellets were ground to a powder in a miniature Waring blender without suspending solvent. The recovered powder was washed with isopropanol on a Buchner funnel, air dried, and sieved to >20 mesh. ProNectin®F (8.0 mg)was dissolved in 8 ml of 10 molar aqueous urea and diluted into 400 ml of 150 mmolar aqueous sodium chloride solution contained in a 1000 ml Erlenmeyer flask.. Polystyrene powder (20 g) was added as a dry powder, and was stirred for 1 hour at room temperature. The recovered polystyrene powder was collected on a Buchner funnel, washed with 100 mM calcium chloride solution, washed with 10 mM calcium chloride solution, sucked as dry as possible, and dried in air

without agitation. The loading of ProNectin®F onto this polystyrene powder was estimated from previous measurements of the surface area of the powder and the adsorption isotherm.

A sample (2.0 g) of polystyrene powder coated with ProNectin®F (200 ppm) was placed into a Pyrex glass tube (16mm X 100mm); sealed with a rubber septum cap, sparged with nitrogen, and heated in a thermostatted oil bath preheated to the required temperature for varying lengths of time. Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150° C and a maximum force of 1500 kilograms for 3-5 seconds. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7 mm "one-hole-punch." A cell attachment assay was conducted on a single 96-well plate using the standard protocol described above.

Table 18.
Optical Densities of Cell Attachment Assay s

	PnF [ppm]	Time [min]	Temp. [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml			8	0.690	±0.050	7%
103,082-A	20	1/4	150°	8	0.691	±0.035	5%
103,082-B	20	5	150°	7	0.610	±0.083	14%
103,082-C	20	40	150°	7	0.554	±0.038	7%
103,082-D	20	10	165°	7	0.561	±0.064	11%
103,082-E	20	5	180°	7	0.517	±0.070	14%
103,082-F	20	20	180°	5	0.330	±0.143	43%
103,082-G	20	10	195°	7	0.364	±0.118	32%
103,082-H	20	40	195°	8	0.204	±0.072	35%
103,082-I	20	5	210°	6	0.348	±0.080	23%
103,082-J	20	10	210°	6	0.205	±0.077	36%
103,082-K	20	20	210°	8	0.106	±0.079	75%

After the polystyrene disks were mounted on the tissue culture plate with silicone grease, a matrix of optical densities was measured. The optical densities reported in the above table are corrected on a well-by-well basis for the variations in optical densities arising from the "cloudiness" of the inserted polystyrene disks and from the silicone grease used to affix the disks to the bottoms of the wells. Each well thus became its own control. The optical densities were also corrected for the fact that

the polystyrene disks tend to pick up a little color during the staining process with the amidoblack.

The performance of the compression molded disks of polystyrene in the cell attachment assay decreased with increasing time and with increasing temperature in a complex manner. These data confirmed that exchange of ProNectin®F on the surface of the polystyrene powder conferred stabilization towards thermal deactivation during the thermal challenge.

The time-temperature experiments represent a response surface which was fitted by multi-variate regression analysis. A model was defined which assumed a semi-first order reaction for thermal degradation with the rate constant being exponential in temperature. The fit of this second equation is $R^2 = 0.969$, and has the satisfying property of fitting only three adjustable parameters to the 11 data points. The following parameters added together to form an equation which can be used to interpolate between points on the time-temperature response surface.

Parameter estimates for $Ln[Ln(A_0/A)]$ $R^2 = 0.969$ Calcium stabilized.

Term	Coefficient	Std Error	t Ratio	Prob>|t|
Intercept	11.878449	1.22902	9.66	0.0000
Ln t	0.3219709	0.04650	6.92	0.0001
1/°K	-6009.332	539.389	-11.14	0.0000

Preferred Embodiments of Coating Process.

The objective of most of the experimentation described herein has been to make a mixture of polystyrene and ProNectin®F and fabricate it into plastic ware useful in tissue culture applications. The fabrication method to be used is injection molding. The injection molding process is characterized by parameters of time, temperature, and mechanical shear stress; all of which determine the activity of the ProNectin®F at the end of the fabrication process. The injection molding process is characterized by parameters of time, temperature, and mechanical shear stress; all of which determine the activity of the ProNectin®F at the end of the fabrication process.

Compression molding may be used as a general method for fabricating plastics into sheets. Compression molding is easier to carry out on a laboratory scale than injection molding. Compression molding combined with the thermal stress experiments was used to model the time and temperature parameters of the injection molding process. The mechanical shear stress parameter can not be readily modeled anywhere except in an actual injection molding experiment.

Compression molding experiments were used to determine how best to mix ProNectin®F with polystyrene. The performance of the best candidate mixture was validated for tolerance to shear stress to an actual injection molding experiment described below.

Grade of Polystyrene

The preferred embodiment uses a grade of polystyrene which contains no mold release agents, lubricants, or viscosity modifiers. Amoco is a major manufacturer of polystyrene. The IR3-C0 grade contains no additives. The GR3-C7 grade contains a mix of additives which are commonly specified for injection molding applications. The detrimental effects of the presence of such additives is shown in the section entitled "Effects of Additives in the Polystyrene", and is summarized below:

Table 19.
Effect of Additives in the Polystyrene.

Sample	PnF [ppm]	Polymer Disks	N	OD [mean]	±s	CV
Bare Plate	Solution coated 1 μg-PnF/ml		8	0.797	±0.037	5%
102,028-2	200	PS [Amoco IR3-C0]	8	0.557	±0.046	8%
102,028-10	200	PS [Amoco GR3-C7	8	0.106	±0.117	111%

Particle Size Of Polystyrene

ProNectin®F will attach to the exposed surface of polystyrene particles. The goal is to achieve an appropriate ratio of μg-PnF per g-PS. One parameter which defines the absolute amount of ProNectin®F which attaches to the polystyrene is the surface area of the polystyrene particles. Small particles possess a greater surface to volume ratio. In general, the use of smaller particles favors absorbing more ProNectin®F per gram of polystyrene. However, it is more difficult to make smaller particles. The preferred embodiment uses polystyrene ground to pass a 20 mesh sieve (>20 mesh). This choice is supported by the relative insensitivity of performance to mesh size over the range of 20 mesh to >100 mesh as shown in the section entitled "Effect of Deposition Methods and Mesh Size", and is summarized below:

Table 20.
Effect of Deposition Method & Mesh Size

Sample	PnF [ppm]	Deposition Method	PS Powder mesh range	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg-PnF/ml			8	0.655	±0.099	15%
103,065-C	150	Vortex Dil'n	>100	8	0.468	±0.112	24%
103,065-F	150	Vortex Dil'n	60-80	8	0.532	±0.071	13%
103,065-I	150	vortex Dil'n	20-40	8	0.410	±0.043	10%

Coating Methods

Evaporative Deposition

In evaporative deposition, a fixed amount of ProNectin®F is dissolved in formic acid solvent, polystyrene powder is added to the solvent, and the solvent is evaporated. All of the ProNectin®F is presumed to be deposited onto the polystyrene powder. A plot of cell attachment activity versus ProNectin®F concentration shows a plateau above 400 ppm. The results are shown in the section entitled "Deposition of ProNectin®F onto Polystyrene by Evaporative Coating", and is summarized below:

Table 21.
Deposition of ProNectin®F onto Polystyrene by Evaporative Coating.

	PnF [ppm]	PS Powder mesh range	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml		8	0.573	±0.024	4%
102-17-03	400	>100	8	0.493	±0.069	14%
103-64-A	400	>100	8	0.461	±0.045	10%
103-64-B	200	>100	8	0.352	±0.086	24%
103-64-C	150	>100	8	0.181	±0.055	30%
103-64-D	100	>100	8	0.115	±0.056	49%
103-64-E	50	>100	8	0.012	±0.049	408%

Coating Methods

Vortex Deposition

In vortex deposition, a fixed amount of ProNectin®F is dissolved in formic acid solvent, polystyrene powder is added to the solvent, and a non-solvent is added

under conditions of vigorous agitation (vortexing). ProNectin®F is deposited onto the polystyrene powder. Under some circumstances, this method may be the preferred embodiment because this method achieved high attachment activities using relatively smaller amounts of ProNectin®F in the coating mixture. A plot of cell attachment activity versus ProNectin®F concentration shows a plateau above 100 ppm. The results are shown in the section entitled "Deposition of ProNectin®F onto Polystyrene by Vortex Dilution Coating", and is summarized below:

Table 22.
Deposition of ProNectin®F onto Polystyrene by Vortex Dilution Coating.

	PnF [ppm]	PS Powder mesh range	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml		8	0.799	±0.026	3%
103-66-00	Solution coated with 100 µl of 1 µg/-PnF/ml		8	0.747	±0.052	7%
103-66-A	300	>20	8	0.516	±0.048	9%
103-66-B	250	>20	8	0.641	±0.073	11%
103-66-C	200	>20	8	0.627	±0.068	11%
103-66-D	150	>20	8	0.650	±0.057	9%
103-66-E	100	>20	8	0.544	±0.067	12%
103-66-F	50	>20	8	0.592	±0.060	10%

Vortex deposition may be conducted using ProNectin®F dissolved in 85% formic acid, 10.0 molar aqueous urea, or 4.5 molar aqueous lithium perchlorate. Dissolution in aqueous urea is the preferred embodiment in the case of vortex dilution because urea is the least toxic or corrosive reagent of this group. These results are shown in the section entitled "Lot Reproductibility & Rinses with Calcium Salts", and are summarized below:

Table 23. Lot Reproducibilities and Rinses with Calcium Salts.

	Comments	Time [min]	Temp [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml			8	0.491	±0.041	1%
103,080-H	Lot #26; Formic; Water Rinse	1/4	150°	7	0.361	±0.132	32%
103,080-I	Lot #26; Formic; $CaCl_2$ Rinse	1/4	150°	8	0.494	±0.043	13%
103,080-J	Lot #26; Urea; $CaCl_2$ Rinse	1/4	150°	8	0.548	±0.036	22%
103,080-K	Lot #26; $LiClO_4$; $CaCl_2$ Rinse	1/4	150°	8	0.519	±0.059	15%

This list of solvent systems is not exhaustive. Other chaotropic reagents may be used to dissolve ProNectin®F in aqueous solutions. Other organic liquids may be used to dissolve the ProNectin®F. Other organic liquids may be used as non-solvents.

Coating Methods

Stirred Deposition

In stirred deposition, ProNectin®F is initially dissolved at "high" concentration (~ 1mg/ml) in a suitable solvent. Preferred solvent is 10.0 M urea. A working solution for coating is prepared by diluting the concentrate down to 5 - 50 µg/ml into 150 mmolar aqueous sodium chloride solution. Coating of the polystyrene is conducted by adding the powdered polystyrene to the working solution and stirring for 1 hour. Three variants were used, which differed in the way the polystyrene powder was prewetted before being added to the working solution. These results are shown in the section entitled "Calcium Stabilization of ProNectin®F", and are summarized below:

Table 24. Calcium Stabilization of ProNectin®F.

	Comments	Time [min]	Temp. [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml			8	0.549	±0.044	1%
103,081-5A	Dry Powder	1/4	150°	8	0.519	±0.065	13%
103,081-3A	Wetted 50% MeOH	1/4	150°	8	0.560	±0.062	11%
103,081-1A	Wetted 100% MeOH	1/4	150°	8	0.531	±0.094	18%
103,081-5B	Dry Powder	5	200°	8	0.172	±0.037	22%
103,081-6B	Dry Powder; Ca^{+2}	5	200°	8	0.418	±0.064	15%
103,081-4B	Wetted 50% MeOH; Ca^{+2}	5	200°	7	0.311	±0.099	32%
103,081-2B	Wetted 100% MeOH; Ca^{+2}	5	200°	7	0.194	±0.052	27%

Based on the results from compression moldings, the preferred embodiment is to use the dry polystyrene powder because this method is easiest to do. The rational for

using dry powder becomes much more compelling when the results from thermal stress and calcium stabilization are considered. In these cases, preparations made using dry powdered polystyrene provide superior performance.

Of the three methods for depositing ProNectin®F onto polystyrene powders, the stirred deposition method is the preferred embodiment. It is the most readily scaled to handling larger quantities of polystyrene.

During the process of stirred deposition, the concentration of ProNectin®F in the working solution determines the amount deposited onto the surface of the polystyrene powder. In order to characterize this deposition phenomenon, we measured the deposition of ProNectin®F onto flat sheets of polystyrene with known surface areas. These results are shown in the section entitled "Relationship of Solution Concentrations to Surface Deposition".

The preferred embodiment is to work at concentrations in the range of 5 - 50 µg-PnF/ml. The most preferred embodiment is to work at 10 - 20 µg-PnF/ml. Other concentrations may become preferred for reasons relating coating costs to the cell attachment performance of the final plastic ware products.

Using the techniques for the Fluorescamine assay described above, ProNectin®F actually deposited onto polystyrene powders was quantitated. Preparation of compression molded film samples and a cell attachment assay were conducted using standard protocols described above. The results of the assay are shown in Table 25.

Table 25. Stirred Deposition of ProNectin®F onto Polystyrene Powders.

	PnF [µg/ml] Solution	PnF [µg/cm^2] Surface	Time [min]	Temp. [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml				8	0.627	±0.014	2%
103-083-A1	40	35.6	1/4	150°	8	0.615	±0.019	3%
103-083-B1	20	26.1	1/4	150°	8	0.599	±0.040	7%
103-083-C1	10	n/d	1/4	150°	8	0.602	±0.021	3%
103-083-D1	5	18.6	1/4	150°	8	0.531	±0.039	7%

Based on these results, the preferred embodiment is to coat polystyrene from a solution concentration of 10 - 20 µg/ml.

Stabilized ProNectin®F

The results of thermal stress experiments are shown in the section entitled "Thermal Stress Matrix for Unstabilized ProNectinF". The first indication that it is

possible to intervene in the thermal deactivation process was observed in the experiments shown in the section entitled "Lot Reproducibility & Rinses with Calcium Salts", as summarized below:

Table 26. Lot Reproducibilities & Rinses with Calcium Salts.

	Comments	Time [min]	Temp [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg/-PnF/ml			8	0.491	±0.041	1%
103,080-H	Lot #26; Formic: Water Rinse	1/4	150°	7	0.361	±0.132	32%
103,080-I	Lot #26; Formic: $CaCl_2$ Rinse	1/4	150°	8	0.494	±0.043	13%

In this experiment, no thermal stress other than that resulting from the compression molding was encountered. The sample which was rinsed with calcium chloride solution performed significantly better than the sample rinsed with water.

The first demonstration that it is possible to intervene in the thermal deactivation process under more severe conditions was observed in the experiments shown in the section entitled "Calcium Stabilization of ProNectin®F", as summarized below:

Table 27. Calcium Stabilization of ProNectin®F.

	Comments	Time [min]	Temp. [°C]	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 μl of 1 μg/-PnF/ml			8	0.549	±0.044	1%
103,081-5B	Dry Powder	5	200°	8	0.172	±0.037	22%
103,081-6B	Dry Powder; Ca^{+2} Rinse	5	200°	8	0.418	±0.064	15%

The calcium chloride solutions were prepared from calcium chloride desiccant which contains titratable base in the amount of 0.006 meq. The presence of the titratable base leads to slightly elevated pH's. The preferred embodiment is to conduct the rinse with calcium chloride solution in two stages: 100 mM $CaCl_2$ followed by 10 mM $CaCl_2$, although in some cases, 1 mM $CaSO_4$ may be substituted for the 10 mM $CaCl_2$ or may be used alone.

The extent to which ProNectin®F may be stabilized towards thermal deactivation is shown in the section entitled "Thermal Stress Matrix for Calcium Stabilized ProNectin®F".

The data points in Table 18 were subjected to multivariate regression analysis, and a equation was derived for predicting deactivation as a function of time and temperature. The general form of this equation was a semi-first order decay with the rate constant exponential in temperature.

The effects of calcium stabilization are apparent when compared against the data in Table 13. Comparing the fitting equations for the calcium stabilized and the non-stabilized cases was instructive. The coefficient on the 1/K° term was interpretable as the energy, E_a/R, of the thermal decomposition reaction, with E_a = ~12 Kcal/mole. Surprisingly, this activation energy does not change, within the confidence intervals, between the two response surfaces. What does change is a decrease in the pre-exponential frequency term Ln[A].

The coefficient on the term in time also changes. The interpretation of this last effect was not clear. It may be related to a surface diffusion phenomenon. In any case, the equations which predict cell culture performance as a function of time and temperature during the thermal challenge are of the form:

$$Ln\left\{Ln\left[\frac{I_o}{I}\right]\right\} = Ln(A) + b * Ln(t) + \frac{E_a}{RT}$$

$$Ln\left[\frac{I}{I_o}\right] = -A * \left[e^{\frac{E_a}{RT}}\right] * t^b$$

$$\frac{I}{I_o} = e^{-A * \left[e^{\frac{E_a}{RT}}\right] * t^b}$$

Where the coefficients have the values:

	Calcium Stabilized		Non-Stabilized	
Terms	Coefficient	Std Error	Coefficient	Std Error
Ln[A]	11.8784	±1.2290	12.2560	±1.4924
b	0.32197	±0.04650	0.70493	±0.08461
Ea	-11,933	±1070	-12,767	±1337
R2		0.969	0.963	

The significance of calcium stabilization, is that we now have a larger time-temperature window through which to conduct the thermo-molding operation. The requirements for the time-temperature window are defined by the characteristics of the thermo-molding process itself. For instance, in the case of injection molding, time is defined by the ratio between the contained volumes within the heated barrel of the screw extruder and within the mold, and by the cycle time on the mold while temperature in defined by the nature of the plastic and the complexity of the mold. Together, these process parameters define "how long" and "how hot" the polypeptide will be stressed.

The preferred embodiment is to ion exchange the ProNectin®F on the surface of the polystyrene with calcium ion at slightly elevated pH, pH 8.6. Other combinations of multivalent metal cations and pH may serve to confer thermal stabilization. Calcium was chosen because it is commonly found in tissue culture media. A small increment of calcium leaching into the tissue culture media from the plastic ware activated with ProNectin®F would constitute only a minimum perturbation on the function of the tissue culture media. Other metal ions which might be useful for stabilization are zinc and magnesium. Trivalent ions such as aluminum may also be useful.

Shear Stress Testing

Native polystyrene pellets (Amoco IR3-P0) were ground to a powder using an 8" vertical grinder and screened to a nominal size of >35 mesh with oversize particles being ~~returned for regrinding. The measured mesh size distribution which was obtained is:~~ (mesh,wt%)<30, 0.2%; 30-35, 11.3%; 35-40, 15.9%; 40-60, 44.9%; 60-80, 18.4%; 80-100, 5.9%; & >100, 3.4%. The polystyrene powder (2000g) was slurried in 3500 ml of isopropanol for 5 minutes, filtered on a Buchner funnel, washed on the filter with an additional 2000 ml of isopropanol, sucked as dry as possible, and dried in air under ambient conditions.

ProNectin®F (500 mg) was dissolved in 500 ml of 10.0 M aqueous urea solution to yield the stock solution of 1 mg/ml concentration. Aqueous saline (150 mM) was prepared by dissolving by sodium chloride (86.5 g) in 10.0 liters of deionized water. The saline solution was agitated using a mechanical stirrer (Lightning model L1UO8P) set at 1025 rpm, the power at 5.8 watts, and the pumping capacity at 365 L/min., with an impeller (model A-310) at the end of the shaft which was angled at 65° to the surface of the solution. Stock ProNectin®F solution (100 ml) was added dropwise over about 3 minutes to the stirred saline to give a final concentration of ProNectin®F of 10μg/ml. To this solution was added 500 g of the ground, washed and dried polystyrene powder >35 mesh. The slurry was allowed to stir for 1 hour at ambient temperature. The polystyrene powder was recovered using a 26 cm Buchner funnel with a stationary porous filter (70 microns). After being sucked as dry as possible, the polystyrene powder was slurried on the funnel with 2 L of 1 mM calcium sulfate solution and again sucked dry. This treatment was repeated twice more. During the final filtration, the filter cake was compacted under an elastic dam. The recovered polystyrene powder was spread into a layer about 3 cm deep and dried for 15 hours in a forced draft oven at 40° C. This oven dried polystyrene powder (750 g) was loaded into a 2000 ml lyophilizer tube, connected to a vacuum line fitted with a trap cooled in liquid nitrogen, and evacuated to 0.008 Torr at 25° C for 1 hour to achieve final drying. The vacuum line was back filled with dry nitrogen to ambient pressure. The lyophilizer tube was removed from the vacuum line, and its access port was sealed with a rubber stopper. The vacuum dried polystyrene powder was stored in these tubes until being poured into the feed hopper of the injection molding machine.

Injection molding using a 1.5 Kg sample of the polystyrene prepared above was conducted using a 5 ounce (141.8 g) TMC injection molding machine. The injection molding was conducted on a cycle time of 25 seconds. The mold used in this test was fashioned in a block of P20 semi-hardened tool steel with a disk shaped cavity of dimensions 1.188" diameter x 0.060" thickness using a 15° draft angle. The gate between the runner and the cavity was designed to exacerbate sheer stress effects and had dimensions of 0.040" x 0.060" x 0.030" (height x width x length). A second mold cavity of completely arbitrary design was installed in the mold frame to increase the shot size to 61.5 grams so that the residence time in the barrel was approximately 58 seconds. The temperature profile on the barrel was: nozzle, 375° F; front, 359° F; middle, 360° F, and rear, 347° F.

Disks prepared in this manner from the coated polystyrene powder were optically indistinguishable from disks prepared from native polystyrene pellets (Amoco IR3-C0) as judged by the transparency of the disk portion of the mold. The sensitivity of the ProNectin®F to shear stresses inherent in the injection molding process was judged by regrinding samples of the molded objects and compression molding the reground polystyrene powder into films suitable for inclusion in the cell attachment assay described above. Shear stress is concentrated at the gate to the mold cavity. To judge this effect, samples for regrinding were taken from the runner immediately before the gate to the disk shaped cavity and from the disk shaped cavity. The results of these cell attachment assays are presented in Table 28.

Table 28. Shear Stress Testing During Injection Molding.

	PnF [ppm]	Time	Temp	N	OD [mean]	±s	CV
Bare Plate	Solution coated with 100 µl of 1 µg-PnF/ml			8	0.396	±0.064	16%
103,092-Z	20	0.25	150°C	8	0.463	±0.059	13%
Reground Runner	20	1.0	193°C	8	0.234	±0.075	32%
Reground Disk	20	1.0	193°C	8	0.059	±0.027	46%

The coated powder (103,092-Z) when compression molded in the form of a thin sheet performed equivalent to the solution coated bare plate in the cell attachment assay.

The polystyrene which was recovered after the injection molding also showed activity in the cell attachment assay. Passage of the molten mixture of ProNectin®F and polystyrene through the gate into the disk shaped cavity led to a deterioration in cell attachment activity compared to the activity of the sample reground from the section of the runner immediately before the gate. This implicates shear stress as a mechanism for deactivating the ProNectin®F during the injection molding process. Shear stress can be alleviated through the design of the sprues, runners, gates, and cavities of the mold. The result of this experiment showed that ProNectin®F can undergo both thermal and mechanical stresses inherent in the injection molding process and retain its cell attachment function.

Oligopeptide 92.10 and Attachment Assay

Peptide 92.10 was prepared to have the following sequence:

$(KKKM)\ (GAGAGS)_2\ GAAVTGRGDSPASAAGY\ (GAGAGS)_2$

A known concentration of peptide 92.10 (estimated to be 41% pure by analytical hplc) and calcium sulfate was dissolved in 88% formic acid. Huntsman polystyrene powder 210N0F (10g) was mixed with 8 ml of the formic acid solution of reagents. The polystyrene was loaded into a Buchner funnel and evacuated under a filter dam (Saran Wrap) to remove excess solution. The amount of entrained solution was determined gravimetrically before and after evaporation of formic acid solvent on a vacuum line. Powder samples were transferred from the Buchner funnel into 1 oz. wide mouth jars, and covered with a Kimwipe® tissue afixed with a rubber band. Multiple 1 oz. sample jars were loaded into a 1600 ml lyophilization tube and dried on a vacuum line fitted with a liquid nitrogen cooled trap to a final pressure of 4 m Torr with the samples at room temperature.

Several sample films from each lot of powder were compression molded from 250 mg of coated polystyrene between 304 stainless steel sheets using a Carver press with plattens heated electrically to 150°C. Four sample disks for cell attachment assay were taken from the center of each of three films using a standard 7mm "one-hole-punch." All cell attachment assays were conducted on a single 96-well plate.

Table 30.

Optical Densities of Cell Attachment Assays On Compression Molded PS With Peptide 92.10

	Peptide 92.10	Calcium	N	OD [mean]	±s	CV
ProNectin®F	264 ppm	5 ppm	8	0.435	±0.071	16%
126,031-A	789 ppm	25 ppm	8	0.340	±0.094	28%
126,031-B	297 ppm	15 ppm	8	0.264	±0.054	20%
126,031-C	316 ppm	32 ppm	8	0.258	±0.083	32%
126,031-D	112 ppm	23 ppm	8	0.157	±0.079	50%
126,031-E	51 ppm	21 ppm	8	0.069	±0.043	62%
126,031-F	40 ppm	33 ppm	8	0.073	±0.070	96%
126,031-G	16 ppm	27 ppm	8	0.030	±0.031	100%
126,031-H	0 ppm	26 ppm	8	0.014	±0.027	190%

Peptide 92.10 is clearly active in a compression molded format. It has about 1/4 the activity (26% ± 8%) of ProNectin®F on a weight basis. The specific activity can not be estimated without independently knowing the content of active RGD's.

The compositions and methods disclosed herein reduce the need for solution coating of finished plastic surfaces. The inventions offer substantial improvement over previously used methods for providing surfaces activated with proteins by allowing any molded device to be activated with one or more thermostable proteins simultaneous with the thermomolding process. This single step reduces costs associated with secondary manufacturing processes for deposition of proteins on the surface of thermomolded articles, many of which are solvent based, and provides the ability to produce finished goods at lower unit cost than conventional methods allow. Furthermore, the disclosed compositions and methods provide for the incorporation of thermostable proteins into devices whose shapes (e.g. spherical or otherwise three-dimensional) are not readily amenable to solution coating processes.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Donofrio, David A
Stedronsky, Erwin R

(ii) TITLE OF INVENTION: PROTEIN-ENRICHED THERMOPLASTICS

(iii) NUMBER OF SEQUENCES: 18

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: FLEHR, HOHBACH, TEST, ALBRITTON & HERBERT
(B) STREET: 4 Embarcadero Center, Suite 3400
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94111-4187

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO Not yet assigned
(B) FILING DATE: 08-JUL-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Rowland, Bertram I
(B) REGISTRATION NUMBER: 20,015
(C) REFERENCE/DOCKET NUMBER: FP-58854-PC/BIR

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 781-1989
(B) TELEFAX: (415) 398-3249
(C) TELEX: 910 277299

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Lys Lys Met Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
1               5                   10                  15
```

Ala Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Tyr
20 25 30

Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser
35 40

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Ala Gly Cys
1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Cys Cys Val
1

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Ser Pro Asp
1

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Val Cys Pro Asp
1

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asp Pro Gly Lys
1

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCTACGTAG TTCTGCCACG TCCGGTATGT TTCGAAAAAG CTGCA 45

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

~~GCTTTTTCGA AACATACGGG ACGTGGCAGA ACTACGTAGC GTGCA~~

~~(2) INFORMATION FOR SEQ ID NO:9:~~

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..222

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GGT GCC GGC AGC GGT GCA GGA GCC GGT TCT GGA GCT GGC GCG GGC TCT   48
Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser
  1               5                  10                  15

GGC GCG GGC GCA GGA TCC GGC GCA GGC GCT GGT TCT GGC GCA GGG GCA   96
Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala
             20                  25                  30

GGC TCT GGC GCA GGA GCG GGG TCT GGA GCT GCA CGC TAC GTA GTT CTG  144
Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr Val Val Leu
         35                  40                  45

CCA CGT CCG GTA TGT TTC GAA AAA GCT GCA GGC TAT GGA GCT GGC GCT  192
Pro Arg Pro Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly Ala
     50                  55                  60

GGC TCA GGT GCT GGA GCA GGA AGC GGA GCG                          222
Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala
 65                  70
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser
  1               5                  10                  15

Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala
             20                  25                  30

Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr Val Val Leu
         35                  40                  45
```

Pro Arg Pro Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly Ala
50 55 60

Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala
65 70

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 945 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Asp Pro Val Val Leu Gln Arg Arg Asp Trp Glu Asn Pro Gly Val
1 5 10 15

Thr Gln Leu Asn Arg Leu Ala Ala His Pro Pro Phe Ala Ser Asp Pro
20 25 30

Met Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
35 40 45

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
50 55 60

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr Val Val
65 70 75 80

Leu Pro Arg Pro Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly
85 90 95

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
100 105 110

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
115 120 125

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
130 135 140

Ala Gly Ser Gly Ala Ala Arg Tyr Val Val Leu Pro Arg Pro Val Cys
145 150 155 160

Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly
165 170 175

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
180 185 190

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
195 200 205

```
Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala
    210                 215                 220

Arg Tyr Val Val Leu Pro Arg Pro Val Cys Phe Glu Lys Ala Ala Gly
225                 230                 235                 240

Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                245                 250                 255

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            260                 265                 270

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
        275                 280                 285

Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr Val Val Leu Pro
    290                 295                 300

Arg Pro Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly Ala Gly
305                 310                 315                 320

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                325                 330                 335

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            340                 345                 350

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
        355                 360                 365

Ser Gly Ala Ala Arg Tyr Val Val Leu Pro Arg Pro Val Cys Phe Glu
    370                 375                 380

Lys Ala Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
385                 390                 395                 400

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                405                 410                 415

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            420                 425                 430

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr
        435                 440                 445

Val Val Leu Pro Arg Pro Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly
    450                 455                 460

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
465                 470                 475                 480

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                485                 490                 495

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            500                 505                 510

Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr Val Val Leu Pro Arg Pro
        515                 520                 525
```

```
Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly
    530                 535                 540

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
545                 550                 555                 560

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                565                 570                 575

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            580                 585                 590

Ala Ala Arg Tyr Val Val Leu Pro Arg Pro Val Cys Phe Glu Lys Ala
        595                 600                 605

Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    610                 615                 620

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
625                 630                 635                 640

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                645                 650                 655

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr Val Val
            660                 665                 670

Leu Pro Arg Pro Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly
        675                 680                 685

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    690                 695                 700

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
705                 710                 715                 720

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                725                 730                 735

Ala Gly Ser Gly Ala Ala Arg Tyr Val Val Leu Pro Arg Pro Val Cys
            740                 745                 750

Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly
        755                 760                 765

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    770                 775                 780

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
785                 790                 795                 800

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala
                805                 810                 815

Arg Tyr Val Val Leu Pro Arg Pro Val Cys Phe Glu Lys Ala Ala Gly
            820                 825                 830

Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
        835                 840                 845
```

```
Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    850                 855                 860

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
865                 870                 875                 880

Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Arg Tyr Val Val Leu Pro
                885                 890                 895

Arg Pro Val Cys Phe Glu Lys Ala Ala Gly Tyr Gly Ala Gly Ala Gly
            900                 905                 910

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Met Asp Pro Gly Arg
        915                 920                 925

Tyr Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln Leu Val Trp Cys Gln
    930                 935                 940

Lys
945
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGGGTGCAT CGATCAAAGT AGCTGTTAGC GCCGGACCGT CTGCA 45

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACGTGGCCCA CGTAGCTAGT TTCATCGACA ATCGCGGCCT GGCAG 45

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 228 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

```
(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
      (A) NAME/KEY: CDS
      (B) LOCATION: 1..228

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGT GCC GGC AGC GGT GCA GGA GCC GGT TCT GGA GCT GGC GCG GGC TCT   48
Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser
  1               5                  10                  15

GGC GCG GGC GCA GGA TCC GGC GCA GGC GCT GGT TCT GGC GCA GGG GCA   96
Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala
             20                  25                  30

GGC TCT GGC GCA GGA GCG GGG TCT GGA GCT GCA CCG GGT GCA TCG ATC  144
Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Pro Gly Ala Ser Ile
         35                  40                  45

AAA GTA GCT GTT AGC GCC GGA CCG TCT GCA GGC TAT GGA GCT GGC GCT  192
Lys Val Ala Val Ser Ala Gly Pro Ser Ala Gly Tyr Gly Ala Gly Ala
     50                  55                  60

GGC TCA GGT GCT GGA GCA GGA AGC GGA GCG GGT GCC                  228
Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala
 65                  70                  75

(2) INFORMATION FOR SEQ ID NO:15:

   (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 76 amino acids
         (B) TYPE: amino acid
         (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser
  1               5                  10                  15

Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala
             20                  25                  30

Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Pro Gly Ala Ser Ile
         35                  40                  45

Lys Val Ala Val Ser Ala Gly Pro Ser Ala Gly Tyr Gly Ala Gly Ala
     50                  55                  60

Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala
 65                  70                  75

(2) INFORMATION FOR SEQ ID NO:16:
```

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1018 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met	Asp	Pro	Val	Val	Leu	Gln	Arg	Arg	Asp	Trp	Glu	Asn	Pro	Gly	Val
1				5					10					15	
Thr	Gln	Leu	Asn	Arg	Leu	Ala	Ala	His	Pro	Pro	Phe	Ala	Ser	Asp	Pro
			20					25					30		
Met	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly
		35					40					45			
Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly
	50					55					60				
Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Ala	Pro	Gly	Ala	Ser
65					70					75					80
Ile	Lys	Val	Ala	Val	Ser	Ala	Gly	Pro	Ser	Ala	Gly	Tyr	Gly	Ala	Gly
				85					90					95	
Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly
			100					105					110		
Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly
		115					120					125			
Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly
	130					135					140				
Ala	Gly	Ser	Gly	Ala	Ala	Pro	Gly	Ala	Ser	Ile	Lys	Val	Ala	Val	Ser
145					150					155					160
Ala	Gly	Pro	Ser	Ala	Gly	Tyr	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly
				165					170					175	
Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly
			180					185					190		
Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly
		195					200					205			
Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Ala
	210					215					220				
Pro	Gly	Ala	Ser	Ile	Lys	Val	Ala	Val	Ser	Ala	Gly	Pro	Ser	Ala	Gly
225					230					235					240
Tyr	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly	Ala	Gly	Ser	Gly	Ala	Gly
				245					250					255	

```
Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            260                 265                 270

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
        275                 280                 285

Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Pro Gly Ala Ser Ile Lys
    290                 295                 300

Val Ala Val Ser Ala Gly Pro Ser Ala Gly Tyr Gly Ala Gly Ala Gly
305                 310                 315                     320

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                325                 330                 335

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            340                 345                 350

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
        355                 360                 365

Ser Gly Ala Ala Pro Gly Ala Ser Ile Lys Val Ala Val Ser Ala Gly
    370                 375                 380

Pro Ser Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
385                 390                 395                     400

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                405                 410                 415

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            420                 425                 430

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Pro Gly
        435                 440                 445

Ala Ser Ile Lys Val Ala Val Ser Ala Gly Pro Ser Ala Gly Tyr Gly
    450                 455                 460

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
465                 470                 475                     480

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                485                 490                 495

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            500                 505                 510

Ala Gly Ala Gly Ser Gly Ala Ala Pro Gly Ala Ser Ile Lys Val Ala
        515                 520                 525

Val Ser Ala Gly Pro Ser Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly
    530                 535                 540

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
545                 550                 555                     560

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                565                 570                 575
```

```
Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
            580                 585                 590

Ala Ala Pro Gly Ala Ser Ile Lys Val Ala Val Ser Ala Gly Pro Ser
        595                 600                 605

Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    610                 615                 620

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
625                 630                 635                     640

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                645                 650                 655

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Pro Gly Ala Ser
            660                 665                 670

Ile Lys Val Ala Val Ser Ala Gly Pro Ser Ala Gly Tyr Gly Ala Gly
        675                 680                 685

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    690                 695                 700

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
705                 710                 715                     720

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
                725                 730                 735

Ala Gly Ser Gly Ala Ala Pro Gly Ala Ser Ile Lys Val Ala Val Ser
            740                 745                 750

Ala Gly Pro Ser Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly
        755                 760                 765

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    770                 775                 780

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
785                 790                 795                     800

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala
                805                 810                 815

Pro Gly Ala Ser Ile Lys Val Ala Val Ser Ala Gly Pro Ser Ala Gly
            820                 825                 830

Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
        835                 840                 845

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
    850                 855                 860

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
865                 870                 875                     880

Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Pro Gly Ala Ser Ile Lys
                885                 890                 895
```

Val Ala Val Ser Ala Gly Pro Ser Ala Gly Tyr Gly Ala Gly Ala Gly
900 905 910

Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly
915 920 925

Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly
930 935 940

Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
945 950 955 960

Ser Gly Ala Ala Pro Gly Ala Ser Ile Lys Val Ala Val Ser Ala Gly
965 970 975

Pro Ser Ala Gly Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly
980 985 990

Ser Gly Ala Gly Ala Met Asp Pro Gly Arg Tyr Gln Leu Ser Ala Gly
995 1000 1005

Arg Tyr His Tyr Gln Leu Val Trp Cys Lys
1010 1015

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Arg Lys Gln Ala Ala Ser Ile Lys Val Ala Val Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Lys Lys Lys Met Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser
1 5 10 15

Gly Ala Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly
20 25 30

Tyr Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser
35 40 45

WHAT IS CLAIMED IS:

1. ~~A composition comprising a functional thermostable polypeptide interdispersed within a thermoplastic.~~

2. The composition of Claim 1 wherein said polypeptide is from 3 to about 60 amino acids in length.

3. The composition of Claim 1 wherein said polypeptide has a molecular weight of at least 6,000.

4. The composition of Claim 1 wherein said polypeptide is a synthetic protein polymer.

5. The composition of Claim 4 wherein said protein polymer is ProNectin®F or SLPL3.0.

6. The composition of Claim 1 wherein said plastic is polystyrene, polyethylene, polypropylene, or polymethylmethacrylate.

7. The composition of Claim 1 further comprising a polypeptide thermostability enhancing additive.

8. A composition produced by contacting a plastic with a functional thermostable polypeptide, heating said plastic and said polypeptide to at least 60°C for at least 15 seconds to create a melt, molding said melt to form a functional thermostable polypeptide interdispersed within a thermoplastic.

9. A method for producing a functional thermostable polypeptide interdispersed within a thermoplastic, said method comprising:

forming a mixture of a plastic and a thermostable polypeptide,

heating said plastic and said protein to create a melt,

molding said melt into a predetermined form,

whereby a functional thermostable protein is interdispersed within a thermoplastic.

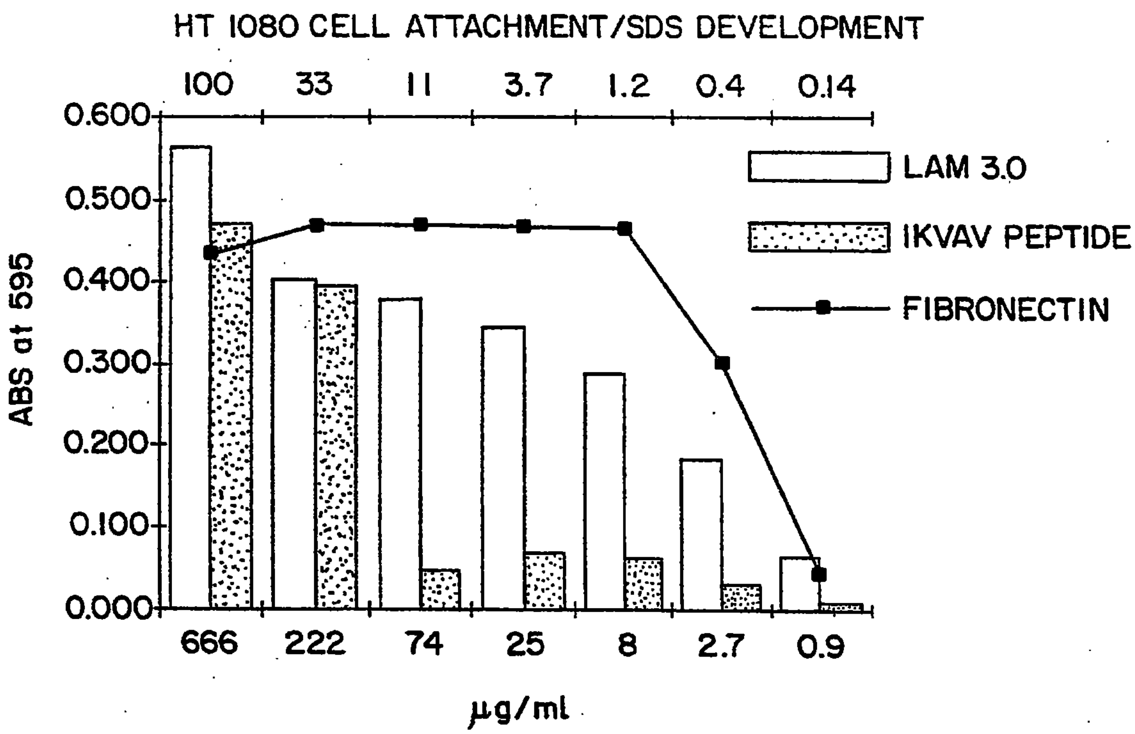
10. A method for producing functional ProNectin®F interdispersed within a thermoplastic, said method comprising:

forming a mixture of a plastic and functional ProNectin®F,

heating said plastic and said ProNectin®F to create a melt,

molding said melt into a predetermined form,

whereby functional ProNectin®F is interdispersed within a thermoplastic.


HT 1080 CELL ATTACHMENT/SDS DEVELOPMENT
100
33
11
3.7
1.2
0.4
0.14
0.600
0.500
0.400
0.300
0.200
0.100
0.000
ABS at 595
LAM 3.0
IKVAV PEPTIDE
FIBRONECTIN
666
222
74
25
8
2.7
0.9
µg/ml

PCT

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(54) Title: PROTEIN-ENRICHED THERMOPLASTICS

(57) Abstract

Thermoplastics interdispersed with a variety of functional thermostable proteins and methods of making such thermoplastics are provided. The disclosure demonstrates that certain proteins can maintain functional integrity through exposure to plastic thermomolding. The proteins are exposed to the heating and molding/extrusion/casting process and are hence present on the formed plastic surface and at a depth below the plastic surface. The proteins contained in the disclosed compositions retain functional properties or binding specificities through the heating and molding/extrusion/casting processes. Preferred thermostable protein used in the disclosed compositions include silk-like protein polymers, particularly ProNectin®F. The disclosed methods and compositions find use in many applications where plastics containing functional thermostable proteins are desired, in particular, cell cultureware.

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AMENDED CLAIMS
[received by the International Bureau on 30 January 1995 (30.01.95); original claims 1-10 replaced by new claims 1-10 (2 pages)]

1. A composition comprising a biologically functional thermostable polypeptide interdispersed within a thermoplastic resulting from a melt at a temperature of at least 60°C, wherein said polypeptide is at least about 25kD, has repeating units from elastin, collagen, keratin or silk proteins and sequences intervening said repeating units having chemically active amino acids or a naturally occurring sequence having binding specificity for a protein receptor.

2. The composition of Claim 1 wherein said intervening group polypeptide is from 3 to about 60 amino acids in length.

3. The composition of Claim 1 wherein said polypeptide has a molecular weight of at least 50kD.

4. The composition of Claim 1, wherein said intervening group comprises RGD.

5. The composition of Claim 4 wherein said protein polymer is ProNectin®F or SLPL3.0.

6. The composition of Claim 1 wherein said plastic is polystyrene, polyethylene, polypropylene, or polymethylmethacrylate.

7. The composition of Claim 1 further comprising a polypeptide thermostability enhancing additive.

8. A formed object produced by contacting a plastic with a biologically functional thermostable polypeptide, heating said plastic and polypeptide to at least 60°C for at least 15 seconds to create a melt, molding said melt to form a biologically functional thermostable polypeptide interdispersed within a

thermoplastic, wherein said polypeptide is at least about 25kD, has repeating units from elastin, collagen, keratin or silk proteins and sequences intervening said repeating units having chemically active amino acids or a naturally occurring sequence having binding specificity for a protein receptor.

9. A method for producing a biologically functional thermostable polypeptide interdispersed within a thermoplastic, said method comprising:

forming a mixture of a plastic and said thermostable polypeptide,

heating said thermoplastic and said polypeptide to create a melt,

molding said melt into a predetermined form,

whereby a biologically functional thermostable polypeptide is interdispersed within a thermoplastic, wherein said polypeptide is at least about 25kD, has repeating units from elastin, collagen, keratin or silk proteins and sequences intervening said repeating units having chemically active amino acids or a naturally occurring sequence having binding specificity for a protein receptor.

10. A method for producing biologically functional ProNectin®F interdispersed within a thermoplastic, said method comprising:

forming a mixture of a thermoplastic and ProNectin®F,

heating said thermoplastic and said ProNectin®F to create a melt,

molding said melt into a predetermined form,

whereby biologically functional ProNectin®F is interdispersed within said thermoplastic.

STATEMENT UNDER ARTICLE 19

In response to the International Search Report for the above captioned PCT application, applicants amend the claims by submitting replacement sheets 69-70, enclosed. Claims 1-10 have been amended to limit the claimed thermoplastics to ones which are produced by a particular process. This limitation further distinguishes the claimed thermoplastics from those products disclosed in the references listed in the search report.